# The Institute of Paper Chemistry

Appleton, Wisconsin

## Doctor's Dissertation

**The Role of Methylglyoxal Glyoxalase in the Growth and Development of Douglas-Fir *[Pseudotsuga Menziesii* (Mirb.) Franco] Needles and Needle Callus**

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**June, 1980**

# THE ROLE OF METHYLGLYOXAL AND GLYOXALASE IN THE GROWTH AND DEVELOPMENT OF DOUGLAS-FIR [PSEUDOTSUGA MENZIESII (MIRB.) FRANCO] NEEDLES AND NEEDLE CALLUS

A thesis submitted by

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of The Institute of Paper Chemistry
for the degree of Doctor of Philosophy
from Lawrence University,
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June, 1980

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"See what everyone else has seen, think what nobody else has thought."

Albert Szent-Gyorgyi

ABSTRACT

Albert Szent-Gyorgyi has suggested (Electronic Biology and Cancer, Marcel Dekker, New York, 1976) that methylglyoxal keeps cells in the resting state from which they can be released by the glyoxalase enzymes for which glutathione is a cofactor. This study has examined that idea by using Douglas-fir needle tissue and Douglas-fir needle callus as representatives of resting (organized) and proliferative (unorganized) states, respectively.

The two glyoxalase enzymes were shown to be present in Douglas-fir needle callus at all times during subculture. Depending on the time since subculture as well as the clone, the glyoxalase I activity varied from 0.55 to 1.40 I.U. (International Units) per gram fresh weight while glyoxalase II varied from 0.10 to 0.65 I.U. per gram fresh weight. Of the two other enzymes known to catabolize methylglyoxal, methylglyoxal reductase and α-ketoaldehyde dehydrogenase, only methylglyoxal reductase was found in the needle callus ranging from 0.20 to 0.30 I.U. per gram fresh weight. Methylglyoxal synthetase activity, which converts dihydroxyacetone phosphate to methylglyoxal, could not be detected in needle callus. This finding is in agreement with the inability to isolate methylglyoxal as the 2,4-dinitrophenylosazone from an insoluble fraction from needle callus.

Glutathione was isolated and identified as the major nonprotein sulfhydryl component in Douglas-fir needle callus. By following the fluctuation of the sulfhydryl content with respect to time it was found that subculturing induced an increase in sulfhydryl levels. Browning of Douglas-fir needle callus, thought to involve oxidation of phenolics to quinones, is accompanied by a decrease in the sulfhydryl content. Since sulfhydryl compounds, in particular glutathinone, are reported to keep phenolics reduced, subculturing may help prevent browning by inducing an increase in sulfhydryl levels.

The situation in Douglas-fir needles was found to be much different with respect to methylglyoxal metabolism. Neither glyoxalase I nor glyoxalase II activity was detected in crude preparations from needle tissue. The needle extract, however, was found to strongly inhibit the glyoxalase I activity from needle callus. Isoelectric focusing of a needle extract separated the inhibitor from the enzyme allowing observable activity. Douglas-fir needles thus contain glyoxalase I activity which is strongly inhibited in crude preparations.

The other enzyme capable of catabolizing methylglyoxal present in needles was methylglyoxal reductase. The activity of this enzyme decreased linearly with age in the needles. The very young primary needles from 1.0 to 1.5 cm contained 0.057 I.U. per gram fresh weight, the fully elongated needles contained no methylglyoxal reductase activity. An evaluation of methylglyoxal synthetase activity during these same stages of needle development showed an activity of 0.097 I.U. per gram fresh weight at the fully elongated stage only. No methylglyoxal synthetase activity was elicited from the very young needles or the mature needles. Furthermore, the isolation of methylglyoxal from an insoluble fraction from Douglas-fir needles as its 2,4-dinitrophenylosazone was accomplished.

If methylglyoxal acts as a natural cell division brake in a normally developing Douglas-fir needle it would appear to involve the proper modulation of methylglyoxal reductase and synthetase activities, as well as the inhibition of glyoxalase I. The presence of glyoxalase I activity in crude

preparation of Douglas-fir needle callus may be the result of its inability to synthesize the inhibitor. In general, the distribution of methylglyoxal and enzyme activities concerned with its metabolism tend to support the cell division hypothesis of Szent-Gyorgyi.

## INTRODUCTION

It is very likely that no other area in all the biological sciences has received more attention than that pertaining to uncontrolled cell division or the cancerous state. The literature that accumulated relative to this subject during the first half of this century is indeed massive. Despite this effort which, for the most part, is merely a collection of a vast array of facts and scattered observations, a very real pessimism pervaded scientific circles. The situation led Peyton Rous, one of the leading researchers of that time, to comment in 1936 that "the tumor problem is the last stronghold of metaphysics in medicine" where one's mind may wander freely formulating new hypotheses on causation (1). The situation changed very little with the advent of molecular biology with its powerful and revolutionary concepts. Therefore, in 1966, Rubin (2) stated: "Thus we have expropriated various bits of information from selected fields of biology, depending on which is the fashion of the day, and applied them with little or no modification to an explanation of how cells become malignant." The lack of success in these efforts may well be attributed to the narrowness of the philosophical base as well as trying to find a cure before an understanding. Either one or the other of these approaches or a combination will invariably lead one in a vicious circle from which there is little chance of achieving success.

However, if the molecular level is transcended to the electronic dimension, one may utilize the wide outlook of natural philosophy to search for a solution. A more logical approach would then be to ask what keeps normal cells from dividing rather than what makes a cancer cell divide. This approach assumes that all cells have an innate capacity and tendency to divide and that a brake must be put on cell proliferation. This type of reasoning forms the basis for Szent-Gyorgyi's cell division hypothesis (3,4,5).

Clues as to the chemical nature of this cell division brake surfaced when it was discovered that some extracts of thymus and liver inhibited the growth of induced cancers while others promoted the growth (6,7). Szent-Gyorgyi went on to name the underlying agent which promoted growth, "promine", and that which retained growth, "retine". While the exact chemical composition of retine was never identified, methylglyoxal was found in the retine extract (8). Retine was thus thought to be methylglyoxal or some derivative of methylglyoxal. This assumption, however, depended on the demonstration that methylglyoxal can actually inhibit cell division in low concentrations in a reversible and specific manner. This was demonstrated by Egyud and Szent-Gyorgyi in 1966 in E. coli (9). Application of 1 mM methylglyoxal completely suppressed cell division without affecting respiration. When the methylglyoxal was decomposed with glyoxalase or inactivated chemically by the addition of cysteine, growth resumed, thereby demonstrating the reversibility of the inhibition.

We may summarize then by assuming along with Szent-Gyorgyi that an α-ketoaldehyde is involved in cellular regulation and that methylglyoxal keeps the cell in its resting state. Having assumed this we must also assume that glyoxalase is kept separated from the methylglyoxal. When a wound, such as a cut, is suffered, glyoxalase is released, acting on methylglyoxal and allowing cell division to occur. When the wound is filled, cell is surrounded by cell, restoring equilibrium and rendering glyoxalase inactive.

The similarity between these arguments and the wounding of trees and formation of callus is obvious. In the event of a wound to a tree the released glyoxalase could inactivate the methylglyoxal and put the cells in

an active state of division for callus formation. Being a mass of rapidly dividing unorganized cells, callus is in many ways similar to cancer and thus may well be subject to similar restraints.

# LITERATURE REVIEW

## GLYOXALASE ENZYME SYSTEM

### HISTORY

The enzymic conversion of methylglyoxal to lactic acid was discovered independently in 1913 by Neuberg (10) and by Dakin and Dudley (11). The activity was noted in a variety of animal tissues and yeast. That same year these investigators proposed the names "glyoxalase" (Dakin and Dudley) and "ketoaldehyde mutase" (Neuberg) for the observed activity (11,12). The rapidity of the reaction was particularly astonishing to Dakin and Dudley (13) who reported that a liver extract could convert twice its weight of methylglyoxal to lactate in 15 minutes if kept neutralized. Lactic acid formation would precipitate the protein if the mixture were not neutralized with powdered chalk. This observation led them to make what was perhaps the first suggestion of a feedback inhibition mechanism for controlling the rate of an enzyme reaction: "The inhibitory effect of acids would seem to be of importance, for it obviously indicates a regulatory mechanism for the action of the enzyme; for both methylglyoxal and the glucose which may yield it are substantially neutral, while an accumulation of the product of the enzyme action, lactic acid, would automatically inhibit further acid formation".

Most of the vast literature about glyoxalase represents the efforts over the years to fit this reaction into the scheme of glycolysis rather than studies of the glyoxalase reaction itself. Stimulated by Cori's (14) work, which had shown that glucose is degraded to lactic acid during muscle activity, attempts were made to ascribe a metabolic role to glyoxalase. Hence, Neuberg and Simon (15) proposed that methylglyoxal may be an intermediate in glucose

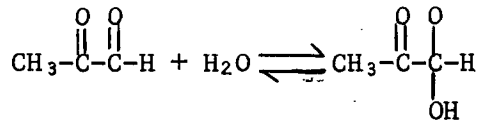
catabolism. Although repeated demonstrations showed appreciable methylglyoxal formation in tissues incubated with intermediates of glucose metabolism, the overall evidence opposed any significant role for methylglyoxal in mammals. Finally Lohmann (16) demonstrated that glycolysis could occur in the absence of glyoxalase activity. Thus, while glyoxalase had demanded the attention of many leading biochemists like Neuberg, Hopkins, Dakin, Lohmann, and Racker in the first half of this century, interest in the enzyme gradually faded out when no glyoxal derivative could be obtained from tissues and no metabolic pathway could be assembled which utilized this substance. The inability to assign the enzyme a physiological function thus led to the study of its reaction mechanism.

## REACTION MECHANISM

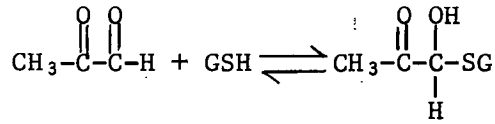
Characterization of glyoxalase activity began with Lohmann (16) who showed the necessity for reduced glutathione in the glyoxalase reaction. This stimulated the proposal by Jowett and Quastel (17) of an intermediate formed by the addition product between methylglyoxal and glutathione prior to lactic acid formation. Subsequent studies by Yamazoye (18) of crude liver extracts showed the existence of an intermediate distinct from the chemical addition complex proposed above. When Hopkins and Morgan (19) demonstrated accelerated lactic acid formation by a protein factor which had no effect on the reaction in the absence of glyoxalase, Racker (20) proposed two separate enzymic steps catalyzed by two separate enzymes. Racker (21) later established that the enzymic conversion of methylglyoxal into lactic acid takes place in two stages. Dakin and Dudley, without citing the evidence, had stated that the reaction was reversible (13). This idea was subsequently revived by Salem and Crook in 1950 (22). However, a subsequent paper by

Salem and Law (23) was in agreement with an earlier conclusion by Racker (21) that formation of the thiolester was irreversible.. The following sequence portrays the reaction scheme as accepted today.

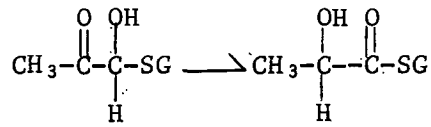
1) methylglyoxal in equilibrium with its hydrated form



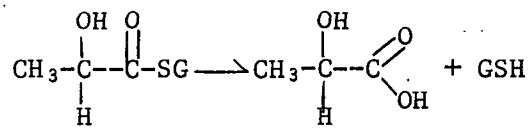
2) chemical addition complex between methylglyoxal and glutathione (nonenzymic)



3) glyoxalase I reaction (intramolecular hydride shift) to form S-lactoyl glutathione



4) glyoxalase II reaction (hydrolysis of thiol ester)



GLYOXALASE I (EC 4.4.1.5) CHARACTERISTICS

Substrate Specificity

Methylglyoxal is the only substrate for glyoxalase I that has been studied in any detail. Other substances shown to be substrates for glyoxalase I include glyoxal, phenylglyoxal, hydroxypyruvaldehyde (24) and phosphohydroxypyruvaldehyde (25). Kethoxal (3-ethoxy-2-oxobutyraldehyde), a cancerostatic agent marketed by Upjohn, is also an active substrate for glyoxalase I while sodium pyruvate, formaldehyde, and diacetyl are not active (26). The reactivities of these ketoaldehydes with purified glyoxalase I using glutathione as coenzyme are shown in Table I.

TABLE I

SUBSTRATE SPECIFICITY OF SHEEP LIVER GLYOXALASE I (26)

| Ketoaldehyde | $V_{max}$(rel), % | $S_{0.5}$ mM | $K_m$ mM |
|---|---|---|---|
| Methylglyoxal | 100 | 0.194 | 0.078 |
| Phenylglyoxal | 85 | 0.147 | 0.113 |
| Kethoxal | 75 | 1.38 | N.D.[a] |
| Hydroxypyruvaldehyde | 15 | 1.54 | N.D. |
| Glyoxal | 2.7 | 0.96 | N.D. |

[a]Not determined.

Coenzyme Specificity

The identification of the role of reduced glutathione (GSH) in the glyoxalase reaction was made in the standard manner of coenzyme discovery. The glyoxalase system required a dialyzable cofactor in order to be active. Lohmann (16) recognized the purified cofactor to be GSH by assaying it in a reconstituted enzyme system. Lohmann further showed that oxidized glutathione (GSSG) could not be substituted for GSH nor could a series of reducing compounds or metal chelators (8-hydroxyquinoline, citrate, pyrophosphate, HCN, and $H_2S$). Other tripeptides were found to be active in the system but were required in higher concentrations than GSH to exert the same effect. Thus Behrens found the $K_m$ values of three active tripeptides to be: GSH, $7.4 \times 10^{-4}$M; asparthione, $3.7 \times 10^{-3}$M; and isoglutathione $1.2 \times 10^{-2}$M (27). No enzyme activity has been obtained with dipeptides or simpler compounds. Substances tested as coenzymes in the glyoxalase I reaction are summarized in Table II.

TABLE II

SUBSTANCES TESTED AS COENZYME IN THE GLYOXALASE I REACTION

| Active | Inactive |
|---|---|
| Glutathione (16) | Thioglycolic acid (16) |
| Isoglutathione (27) | Thioneine (30) |
| Asparthione (27) | Ascorbic acid (30) |
| γ-D-Gluo-L-Cys-Gly (28) | Cysteine (16) |
| S-Acetylglutathione (29) | Oxidized Glutathione (16) |
| α-L-Glutamyl-L-cysteinyl-glycine (27) | Cysteinylglycine (27) |
| | γ-Glutamylcysteine (31) |
| β-L-Aspartyl-L-cysteinyl-glycine (27) | S-Methylglutathione (32) |
| | N-Acetylcysteine (26) |
| | Mercaptoethanol (26) |
| | Dithiothreitol (26) |
| | Thiomalate (26) |

## Inhibitors

Studies dealing with inhibitors of glyoxalase I activity have concentrated on the effect of glutathione analogues. Carbonyl compounds such as formaldehyde can inhibit the rate of glyoxalase I activity with methylglyoxal as substrate by reacting with glutathione and tying up the cofacter (26). However, high concentrations of formaldehyde, for example, are required. The inhibitory effects of various glutathione analogues on the glyoxalase I reaction are listed in Table III.

## Purification Data

The difficulty experienced in obtaining a homogeneous preparation of glyoxalase I has only recently been overcome. Racker (21) was able to obtain

a partial purification from yeast in 1952 and Davis and Williams obtained a 200 fold purification from calf liver in 1969 (34). The latest attempts at purification have led to homogeneity for glyoxalase I (26). Purification schemes described in the literature employ the conventional techniques of column chromatography, affinity chromatography, electrophoresis, and isoelectric focusing. Table IV summarizes recent glyoxalase I purification data.

TABLE III

EFFECT OF GSH ANALOGUES ON THE GLYOXALASE I REACTION

| Inhibitor | $K_i$ (mM) | Apparent $K_m$ (mM) | Type of Inhibition | Ref. |
|---|---|---|---|---|
| S(N-Ethylsuccinimido) glutathione | 0.64 | 0.55 | Competitive | (32) |
| S-Methylglutathione | 2.3 | 0.67 | Competitive | (32) |
| γ-DL-glutamyl-DL-alanylglycine | 15.1 | 8.3 | Intermediate | (32) |
| γ-L-glutamyl-L-B-sulphoalanylglycine | 18.7 | 0.43 | Competitive | (32) |
| γ-DL-glutamylglycyl-glycine | 40.9 | | Noncompetitive | (32) |
| S-methyl-L-cysteine | 22.5 | 9.3 | Intermediate | (32) |
| Opthalmic acid | 0.94 | N.D.[a] | Competitive | (33) |

[a]Not determined.

Glyoxalase I shows a rather broad pH optimum of activity from 6 to 8 and is quite stable if kept frozen at -20°C (21). The enzyme has been kept frozen for over one year without appreciable loss of activity. Dilute solutions of the enzyme are quite unstable and must be protected with bovine serum albumin. Enzyme activity is maintained below 60°C while temperatures above

65°C lead to rapid inactivation. The enzyme is very rapid with a turnover number of 40,000 (4). The isoelectric point of metal free apoglyoxalase I from sheep liver was found to be 5.0 by isoelectric focusing (26). The amino acid composition is consistent with the low isoelectric point (26,35).

TABLE IV

GLYOXALASE I PURIFICATION DATA

| Source | Specific activity (IU/mg protein) | Molecular Weight | Subunit Molecular Weight | Purification Factor | Ref. |
|---|---|---|---|---|---|
| Mouse Liver | 2200 | 43000 | N.D.[a] | 1140 | (35) |
| Sheep Liver | 4000 | 45900 | 21000 | 5000 | (26) |
| Pig Erythrocytes | 957 | 48000 | 24000 | 11500 | (36) |

[a]Not determined.

It has been noted that glyoxalase I from all sources investigated is inactivated by treatment with metal chelators such as EDTA (26,34,36). This indicates that the enzyme is dependent on metal ions for enzymic activity. The enzyme from calf liver was found to be reactivated after addition of $Mg^{+2}$ (34) and this observation has been extended to a variety of sources (26,36). While other divalent metal cations were capable of restoring activity to varying degrees, $Mg^{+2}$ was the most efficient and was assumed to be the natural cofactor. A recent report, however, demonstrates that glyoxalase I from human and porcine erythrocytes, rat liver, and yeast contains zinc which is essential for the enzymic activity (37).

Racker made the observation in 1952 that a partially purified preparation of glyoxalase I frequently displayed activity in the absence of added glutathione (21). He felt this indicated the possibility that glutathione was associated with the enzyme. Recent studies have shown that the purified

enzyme can be resolved into two catalytically active components by polyacrylamide gel electrophoresis (36,38). In these cases the two components can be fused into one species by preincubation with glutathione. It has thus been proposed that glyoxalase I exists in two interconvertible forms one of which is a mixed disulfide with glutathione (36).

The reaction rate for glyoxalase I is linearly dependent on enzyme concentration under saturating substrate conditions. The initial rate of formation of the hemimercaptal is proportional to the concentration of methyglyoxal but independent of the glutathione concentration (33). It is believed that the rate-determining step in the formation of the hemimercaptal is the dehydration of methylglyoxal. Thus at low methylglyoxal concentrations and high enzyme concentrations the rate of the reaction is affected by the rate of the nonenzymic formation of hemimercaptal.

## GLYOXALASE II (EC 3.1.2.6) CHARACTERISTICS

### Substrate Specificity

The number of studies in the literature that have examined glyoxalase II activity are rather limited. The glyoxalase II reaction appears, however, to be rather specific for glutathione but rather unspecific for the acyl group of the thiol ester. A report by Uotila (39) appears to be the most comprehensive to date. The data showing the reactivity of glyoxalase II toward nine glutathione thiol esters are shown in Table V. Although S-lactoylglutathione had the greatest maximum velocity, S-mandelolyglutathione had the lowest $K_m$ value.

### Inhibitors

Schwyzer and Hurlimann (40), on the basis of studies with organic models, have suggested the importance of metals in reactions in which thiol esters

are involved. When added to glyoxalase II preparations, however, EDTA has no instantaneous effects (39). Only after long term storage will 1 mM EDTA cause labilization of the enzyme. Attempts at reactivation of such a system with a series of divalent metal cations does not restore activity. Metal salts such a $MgSO_4$, $MnSO_4$, $NiCl_2$, and $Co(NO_3)_2$ at 1 mM have no effect (39). NaCl, KCl, and $NH_4Cl$ give some inhibition above 15 mM. Phosphate at 67 mM inhibits the activity 35% while 167 mM inhibits 65%. Similar effects are observed for arsenate. Pyrophosphate meanwhile inhibits 20% at 5 mM and 50% at 25 mM.

TABLE V

MICHAELIS CONSTANTS AND MAXIMUM VELOCITIES OF SUBSTRATES OF PURIFIED GLYOXALASE II (39)

| Substrate | $K_m$ (mM) | $V_{max}$ (rel) |
|---|---|---|
| S-lactoylglutathione | 0.190 | 100 |
| S-glyceroylglutathione | 0.109 | 61.8 |
| S-glycoylglutathione | 0.070 | 38.6 |
| S-mandeloylglutathione | 0.016 | 4.8 |
| S-acetoacetoylglutathione | 0.295 | 55.6 |
| S-succinoylglutathione | 0.200 | 29.2 |
| S-formoylglutathione | 0.153 | 37.7 |
| S-acetoylglutathione | 0.266 | 8.6 |
| S-propionoylglutathione | 0.213 | 13.5 |

The glutathione analogues S-methylglutathione and S-hexylglutathione at 1 mM and 0.1 mM, respectively, as well as formaldehyde and acetaldehyde, each at 10 mM, are without effect (39). Glutathione is a rather weak inhibitor with a $K_i$ value of 4.0 mM while the spontaneously formed methylglyoxal-glutathione hemimercaptal shows a marked inhibition. DL-Lactate at 70 mM does not

exhibit any inhibition. The sulfhydryl inhibitor $HgCl_2$ does inhibit glyoxalase II activity and the enzyme can be partially reactivated with dithiothreitol. The high concentrations of sulfhydryl reagents necessary to cause inhibition, however, oppose the idea of a sulfhydryl group at the enzymes active site (39).

Purification Data

Most of the attention concerning purification and characterization of the glyoxalase system has been focused on glyoxalase I. Thus the properties of glyoxalase II from a variety of sources is not well established. Purifications of this enzyme of only 20 to 30 fold have been reported (21,41,42). Jerzykowski et al. (42) were the first to report the molecular weight of glyoxalase II from beef liver and chicken kidney as 23,000. The specific activity of this preparation was reported as 20 I.U./mg protein. More recently Uotila (39) has obtained a 4300 fold purification from human liver with a specific activity of 822 I.U./mg. The molecular weight of glyoxalase II from this preparation was reported as 22,900.

The optimum activity occurs over the pH range from 6.8 to 7.5. The activity decreases rapidly above pH 7.5 due to dissociation of the active site, while loss of activity below 6.8 is gradual until denaturation at pH 4.0 (39). Glyoxalase II is much more temperature sensitive than glyoxalase I. At neutral pH the enzyme is stable only up to 35°C with loss of half activity at 45°C and all activity at 60°C. The purified enzyme can be stabilized with bovine serum albumin, glycerol, mercaptoethanol, and dithiothreitol (39). The reaction rate of glyoxalase II is linearly dependent on both enzyme and substrate with S-lactoylglutathione (39).

## DISTRIBUTION IN LIVING SYSTEMS

### Animals

For many years after the time of its discovery in 1913, the glyoxalase system was studied mainly in human and animal tissues. Glyoxalase activity was demonstrated in a variety of these tissues by determining the rate of $CO_2$ evolution from sodium bicarbonate solutions due to lactic acid formation. The most comprehensive study on the distribution of glyoxalase activity during this period was reported by Hopkins and Morgan in 1945 (43). These early studies were all performed before it was known that the glyoxalase system is composed of two enzymes. Thus the activity of the glyoxalase system in these tissues was determined by assessing the overall reaction. When low glyoxalase activity was observed, it was impossible to conclude which of the enzymes was really absent or decreased. With Racker's development of a spectrophotometric assay for glyoxalase, the activities could be ascertained individually (21). A reexamination of the distribution of the glyoxalases in normal and neoplastic tissues has been presented recently (44). The results indicate the universal distribution of both glyoxalase I and II in animal tissues and the absence or lower activity of these enzymes in neoplastic tissues and cells. A portion of these data are represented in Table VI.

It is important to note that although certain neoplastic tissues show no glyoxalase I or glyoxalase II activity, they are still capable of metabolizing methylglyoxal. In Yoshida sarcoma cells, for example, where no glyoxalase II activity is observed, a marked rate of methylglyoxal disappearance is observed (44) when assayed by the colorimetric method (45).

TABLE VI

GLYOXALASE I AND II ACTIVITY IN VARIOUS NORMAL AND TUMOR TISSUES AND CELLS (44)

| Source | G-I[a] | G-II | Liver | | Kidney | | Brain | |
|---|---|---|---|---|---|---|---|---|
| | | | G-I | G-II | G-I | G-II | G-I | G-II |
| Man (Homo-sapiens) | - | - | 26±7 | 108±4 | 40±6 | 99±26 | 138±23 | 245±47 |
| Pig (Susscrofa) | - | - | 178±45 | 163±10 | 278±53 | 54±9 | 274±36 | 80±4 |
| Rabbit (Orycto-lagus cuniculus) | - | - | 447±91 | 149±23 | 131±13 | 129±4 | 117±35 | 86±2 |
| Guinea Pig (Cavia cutleri) | - | - | 178±22 | 423±66 | 94±31 | 374±49 | 176±33 | 229±51 |
| Mouse (Mus musculus) | - | - | 233±56 | 124±38 | 182±52 | 104±21 | 192±47 | 68±9 |
| Ehrlich Carcinoma Cells (Mouse) | 30±9 | 26±5 | | | | | | |
| Yoshida Sarcoma Cells (Rat) | 18±1 | 0 | | | | | | |
| Carcinoma Hepato-cellulare (Human) | 0 | 0 | | | | | | |
| Guerin Tumour (Rat) | 63±8 | 0 | | | | | | |
| Sarcoma 180 Tumour (Mouse) | 97±11 | 25±6 | | | | | | |

[a]Results are given in units/mg of protein as means ± S.E.M. Assayed by method of Racker (21).

Plants

Dakin and Dudley (11) were the first to observe glyoxalase activity in plant systems. They made the general observation that glyoxalase levels were lower in plants than in animals. Later Neuberg and Kobel (46) demonstrated the enzymes presence in green leaves. In 1945 Hopkins and Morgan studied a number of different plant species for glyoxalase activity (43). On the basis

of their observations these workers concluded that glyoxalase is widely distributed throughout the plant world. Of extreme importance and relevance to this thesis is the conclusion by Hopkins and Morgan that conifers did not contain glyoxalase activity. Young spring growth was assayed in their study and no glyoxalase activity was elicited. Thus conifers and brown seaweeds seemed unique among those plants analyzed to date in their lack of glyoxalase activity. Some of the plant species analyzed by Hopkins and Morgan for glyoxalase activity are shown in Table VII. Although the conifers and brown seaweed gave positive values for $CO_2$ evolution, they were so small that, in their estimation, no glyoxalase was present (43). Experience with the system led them to the conclusion that in those cases where the evolution of $CO_2$ was very low some nonenzymic chemical reaction was responsible. Nevertheless, controls were not presented which validate this judgment.

## POSSIBLE METABOLIC ROLES

The physiological function of the glyoxalase system currently remains a mystery in spite of the fact that 66 years have elapsed since the time of its discovery. A concerted effort for the first thirty years to associate the glyoxalase system with glycolysis was unsuccessful. Hopkins and Morgan expressed the desire in 1945 that their experiments extending the range of glyoxalase distribution would encourage further work in the field. It seems very unlikely that evolution would allow such an active and widely distributed protein to be transcribed for no reason.

Many times by studying the distribution of enzyme activity in tissues, information on its metabolic function can be derived. The observed distribution of glyoxalases among various animals and tissues, however, does not give any strong hints as to its biological role. Jerzykowski et al. have

stressed the point that the lack of or decrease of glyoxalase II in certain tumour tissues and cells could be of importance in the process of carcinogenesis (44). Aronsson and Mannervik meanwhile have suggested a detoxification function for the glyoxalases to protect against the methylglyoxal formed by enterobacteria in the alimentary canal (36). The broad specificity of the glyoxalases for many α-ketoaldehydes is certainly consistent with a detoxification role.

TABLE VII

GLYOXALASE ACTIVITY OF ALGAE, FUNGI, AND HIGHER PLANTS (43)

| Order | Species | Name | Part of Plant | μL $CO_2$ Evolved | |
|---|---|---|---|---|---|
| | | | | 20 min | 60 min |
| Algae | *Cystoclomium* | Seaweed (red) | - | 84.0 | 203.0 |
| Algae | *Enteromorpha* | Seaweed (green) | - | 60.5 | 162.2 |
| Algae | *Laminaria digitta* | Seaweed (brown) | - | 21.0 | 33.0 |
| Fungi | *Penicillium notatum* | Mould | - | 36.0 | 102.0 |
| Fungi | *Psalliota campestris* | Mushroom | - | 156.0 | 375.0 |
| Coniferae | *Cypress* | Cypress | - | 27.0 | 39.0 |
| Coniferae | *Taxus baccata* | Yew | - | 15.0 | 18.0 |
| Coniferae | *Pinus* | Young Pine Cones | - | 15.0 | 18.0 |
| Solanaceae | *Solanum tuberosum* | Potato | Leaves | 84.0 | 222.0 |
| Compositae | *Lactuca sativus* | Lettuce | Leaves | 63.2 | 178.7 |
| Cruciferae | *Brassica oleracea* | Cabbage | Leaves | 93.5 | 225.5 |

An additional role has been ascribed to the glyoxalases by Nobel Laureate biochemist Szent-Gyorgyi (3,4,5,9,47-51). According to his hypothesis, methylglyoxal or some derivative of methylglyoxal acts as a brake on cellular proliferation by keeping cells in the resting state. Glyoxalases would inactivate the ketoaldehyde thereby releasing the brake on cell division and allowing cellular proliferation to proceed. The role of methylglyoxal could be determined by the presence of an active or inactive form of glyoxalase. This hypothesis was central to this thesis.

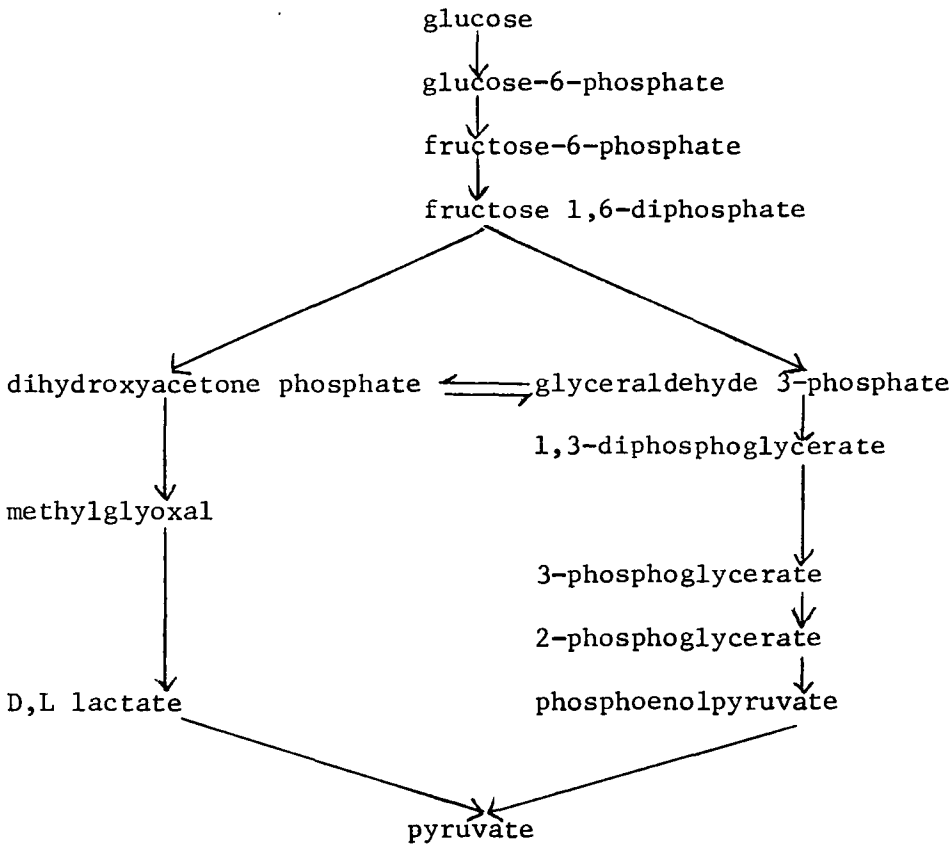
# METHYGLYOXAL

## MODES OF SYNTHESIS IN LIVING SYSTEMS

The mechanism of *in vivo* formation of methylglyoxal has long been an elusive subject. While many studies claimed that methylglyoxal was formed by glycolyzing tissues (52-54), its formation was later considered artifactual and its role in glycolysis dismissed by Meyerhof (55). Meyerhof and Lohmann (56) had demonstrated the conversion of triose phosphates to methylglyoxal in a nonenzymic reaction catalyzed by acid. More recently Riddle and Lorenz (57) showed that methylglyoxal could be formed nonenzymically from dihydroxyacetone and DL-glyceraldehyde with a variety of catalysts. The published reports of methylglyoxal formation in glycolyzing tissues were thus thought to occur by these nonenzymic side reactions.

Since methylglyoxal formation was considered to be due to nonenzymic side reactions of trioses its importance was deemed trivial. However, Cooper and Anderson, in 1970 reported an enzyme from *Escherichia coli* which catalyzed the formation of methylglyoxal from dihydroxyacetone phosphate (58). The enzyme was named methylglyoxal synthetase and has since been isolated from *Proteus vulgaris* as well (59). Preliminary studies on the presence of the enzyme in erythrocytes were negative (36). Recently, Alexeev *et al.* (60) have reported that tumour homogenates do not contain the enzymatic system involved in methylglyoxal formation while muscle homogenates do. Currently then, the distribution of methylglyoxal synthetase reported in the literature is limited to these bacteria and muscle extracts. The synthesis of methylglyoxal and its relation to glycolysis is depicted in Scheme I. Since the diversion of triose phophate to pyruvate through methylglyoxal

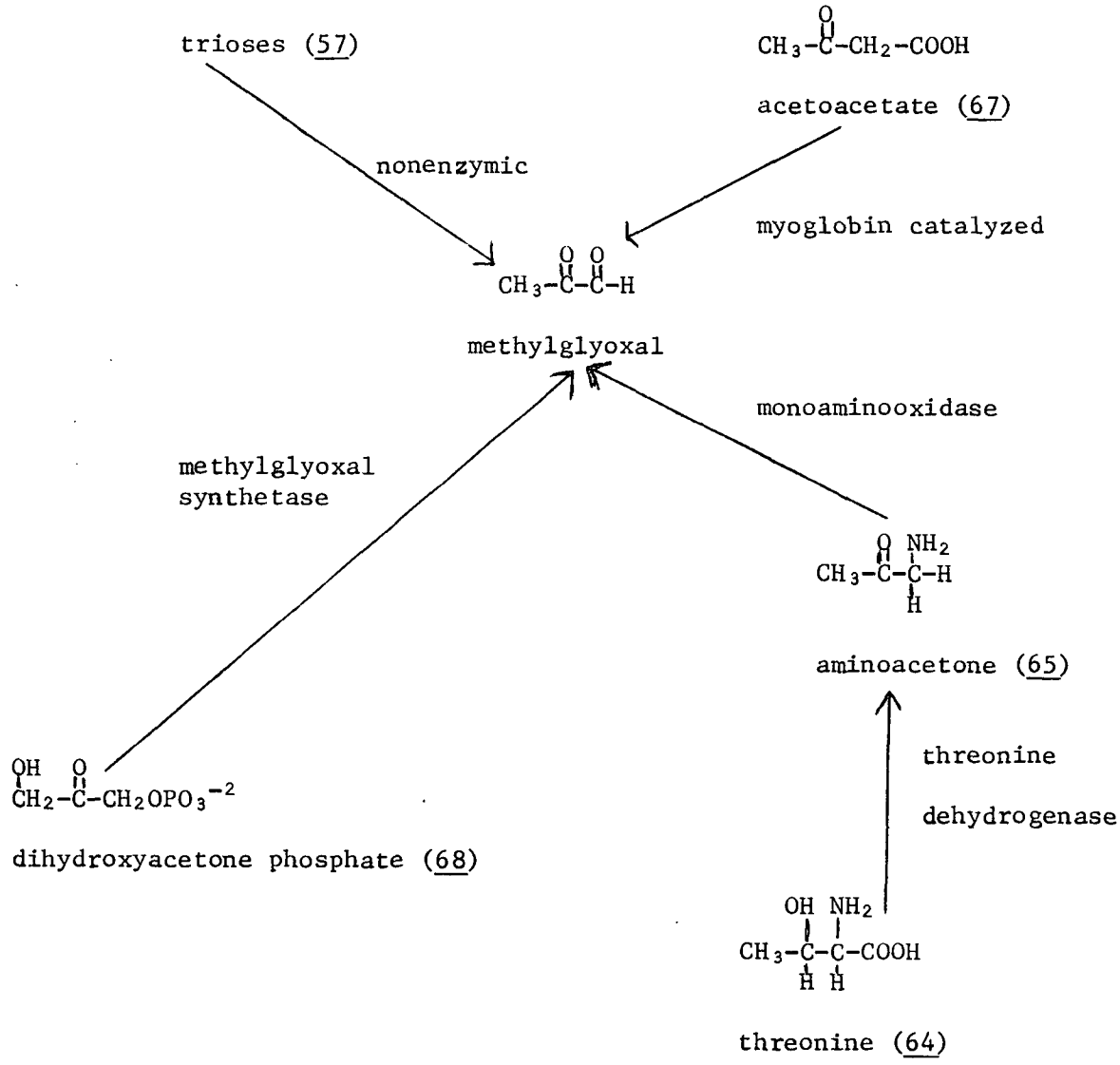
would not be coupled to ATP synthesis, this route may play a role in the uncoupling of catabolism and anabolism which has been recognized for many years (61).




Scheme I. Outline of the Embden-Meyerhof Glycolytic Pathway Showing the Methylglyoxal By-Pass (62)

The properties of a purified preparation of methylglyoxal synthestase from E. coli have been reported by Cooper and Hopper (63). They obtained a 1500 fold purification and achieved a specific activity of 500 units/mg protein. The purified enzyme could be inactivated by heat or proteolysis and had a molecular weight of 67,000. Optimum pH was 7.5 and the enzyme was specific for dihydroxyacetone phosphate with a $K_m$ of 0.47 mM. Phosphenolpyruvate, 3-phosphoglycerate, $PP_i$, and $P_i$ were potent inhibitors of the enzyme activity.

An additional means of methylglyoxal formation is through the catabolism of threonine. Threonine can be converted to aminoacetone by means of the threonine dehydrogenase reaction (64). Monoaminooxidase then converts aminoacetone to methylglyoxal (65). These reactions have been reported in Bacillus subtilis catabolism of threonine (66). Scheme II depicts the various pathways known for the synthesis of methylglyoxal.




Scheme II. Methylglyoxal Synthesis Pathways

## MODES OF DEGRADATION IN LIVING SYSTEMS

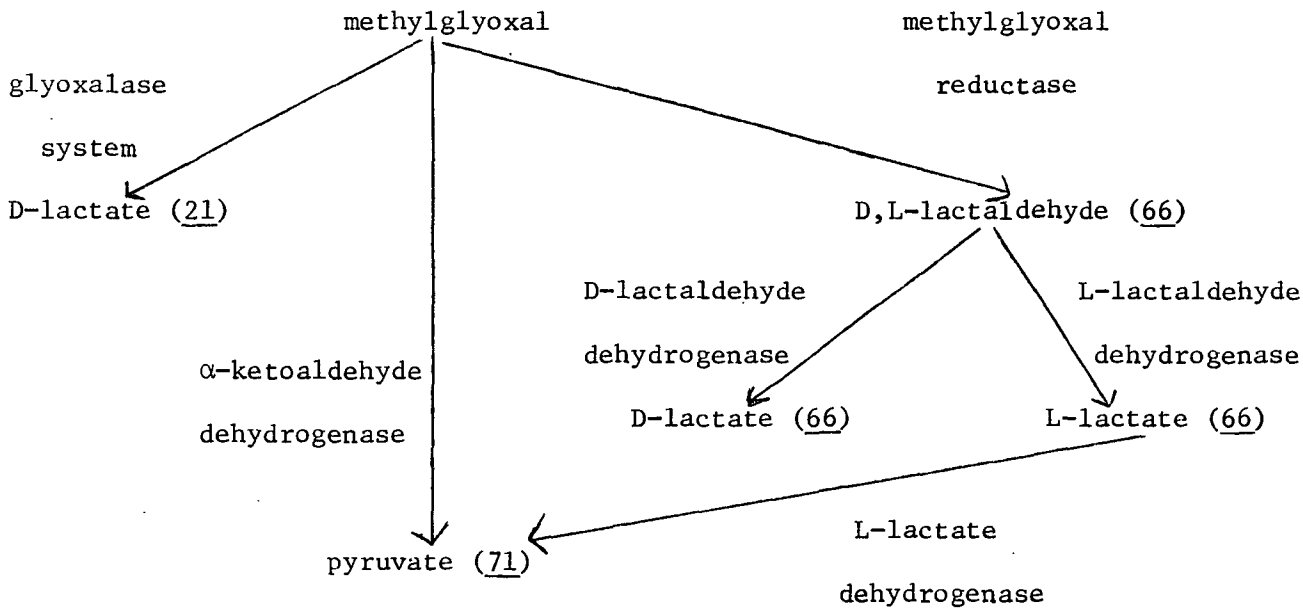
The pathway generally considered for the catabolism of methylglyoxal is through D-lactate by the glyoxalase system. An isomerase (69) was then suggested as the means of converting D-lactate to L-lactate, a utilizable form. The isomerization of D-lactate, however, has not been convincingly demonstrated and D-lactate is poorly utilized by animals. Evidence of this has been reported by Van Eys et al. (70) who found that in rats suffering from thiamine deficiency methylglyoxal levels in the urine increased as did the D- to L-lactate ratio. This suggested the action of glyoxalase as responsible for the formation of D-lactate which then tended to accumulate due to the lack of the isomerase.

More recently, however, two other enzyme systems have been shown to exist which metabolize methylglyoxal. Monder in 1967 reported an enzyme from sheep liver which catalyzed the oxidation of methylglyoxal directly to pyruvate using $NAD^+$ or $NADP^+$ (71). The enzyme was named α-ketoaldehyde dehydrogenase and has since been reported to exist in B. subtilis (66) and rat liver (72). A third route for the catabolism of methylglyoxal is via lactaldehyde due to the action of methylglyoxal reductase (66). This enzyme which requires either NADH or NADPH has also been found in B. subtilis (66). The catabolism of methylglyoxal by known enzymatic routes is outlined in Scheme III.

## EXISTENCE IN LIVING SYSTEMS

A variety of early studies claimed that methylglyoxal was formed by glycolyzing tissues (52-54). However, its formation was subsequently considered to be artifactual and its role in glycolysis dismissed by

Meyerhof (55). Other investigators have reported an accumulation of methylglyoxal in thiamine deficient animals (73,74). These reports have likewise been dismissed on the basis of data which provides conclusive evidence that methylglyoxal does not accumulate in the thiamine deficient rat (75). The existence of methylglyoxal in E. coli has been demonstrated under certain physiological conditions (62). These conditions produce an excess of methylglyoxal and it can be detected in the medium.




Scheme III. Enzymes of Methylglyoxal Catabolism

Evidence for the existence of methylglyoxal in higher plant and animal systems is very limited. Egyud in 1965 found methylglyoxal in a retine extract from the thymus gland (8). Fodor et al. reported in 1978 the isolation of methylglyoxal from a structural protein extract of beef liver (76). In plants, methylglyoxal has been reported to be a component of coffee aroma (77).

EFFECT ON CELL DIVISION

Recent studies of methylglyoxal have focused on the possible physiological function of this compound as a naturally occurring inhibitor of cell division (3,4,5,9). It is well known that methylglyoxal possesses a very stron cancerostatic action (78-80). Methylglyoxal and its derivatives have been found to inhibit the cell division of bacteria, fertilized sea urchin eggs, germinating seeds, flagellates, and mammalian cells in culture (9). This was demonstrated with low concentrations of methylglyoxal and was found to be reversible by the addition of cysteine. Thus 1 mM methylglyoxal completely suppressed cell division in E. coli without affecting respiration. Szent-Gyorgyi et al. (47) stated that inhibition of cell division was due to inhibition of protein synthesis at the ribosomal level rather than interference with nucleic acid synthesis. It was later confirmed by Gregg (81) that these compounds inhibit protein synthesis in cultured mammalian cells.

Morris (82) has reported the inhibition of the growth and cell division of green algae with methylglyoxal concentrations of 1.0-2.0 mM. He showed that protein synthesis was inhibited at less than 1.0 mM methylglyoxal, while polysaccharide synthesis was actually stimulated. At higher concentrations (1.5-2.5 mM) both processes were inhibited.

A more thorough study of the effects of methylglyoxal on higher plants was reported in 1974 by Mankikar and Rangekar (83). Barley seeds were allowed to imbibe varying concentrations of methylglyoxal and observed for germination. Percent germination, fresh weight, protein and RNA levels were determined at six hour intervals. Results indicated inhibition of germination proportional to methylglyoxal concentration. While seeds subjected to $10^{-4}$M and less recovered after eight hour lag times, inhibition was complete at $10^{-3}$M

methylglyoxal. Since the addition of cysteine or methionine in the imbibing solution counteracted the inhibition due to methylglyoxal at $10^{-4}$M and less, the involvement of sulfhydryl groups of key enzymes was implicated.

# GLUTATHIONE

## INTRODUCTION

Living systems all require sulfur in one form or another. The sulfur bacteria, for example, utilize free sulfur, plants can use sulfate or sulfide, and animals make use of cystine and methionine residues of proteins. Regardless of the form of sulfur ingested by these organisms it appears intracellularly in three principal groups which are somewhat interconvertible.

Methionine residues from proteins make up the sulfur fraction which is characterized by $-S-CH_3$ groups. A variety of polysaccharides and steroids contain sulfur as sulfate esters and amides which comprises the second sulfate fraction. The third fraction is composed of cellular free sulfhydryl or thiol (SH) groups and oxidized or disulfide (SS) groups.

The reduced sulfur compounds may be further subdivided into the protein and nonprotein thiols. Glutathione (GSH) is the most abundant nonprotein thiol found in living systems. The one proven exception to this statement is found in bean seedlings where the major nonprotein thiol is homoglutathione (84).

Glutathione is a tripeptide consisting of the three amino acids, glutamic acid, cysteine, and glycine, respectively. This compound is somewhat peculiar in that the glutamic acid is linked through its gamma carbon atom to cysteine. The sulfhydryl moiety from the cysteine, however, is what lends the molecule its reactivity. Glutathione can exist in reduced form as GSH or in oxidized form as GSSG. Autoxidation will not occur, however, unless a catalyst such as trace metal ions, metal complexes, or selenite are present.

MODE OF SYNTHESIS IN LIVING SYSTEMS

Plant cells must possess the ability to both synthesize and degrade glutathione since it is the main cellular nonprotein thiol. Being a tripeptide, the biosynthesis of glutathione is concerned chiefly with the assembly of its three amino acid constituents, glutamic acid, cysteine, and glycine. The synthesis is known to occur in two steps, each requiring a separate enzyme. Each enzyme requires ATP and $Mg^{+2}$ for its activity while in plants the rate is increased by $K^{+}$. The reaction scheme for the biosynthesis of glutathione is shown in Scheme IV. In beans the glycine in the second enzymatic step is replaced by alanine thus making homoglutathione in place of glutathione.

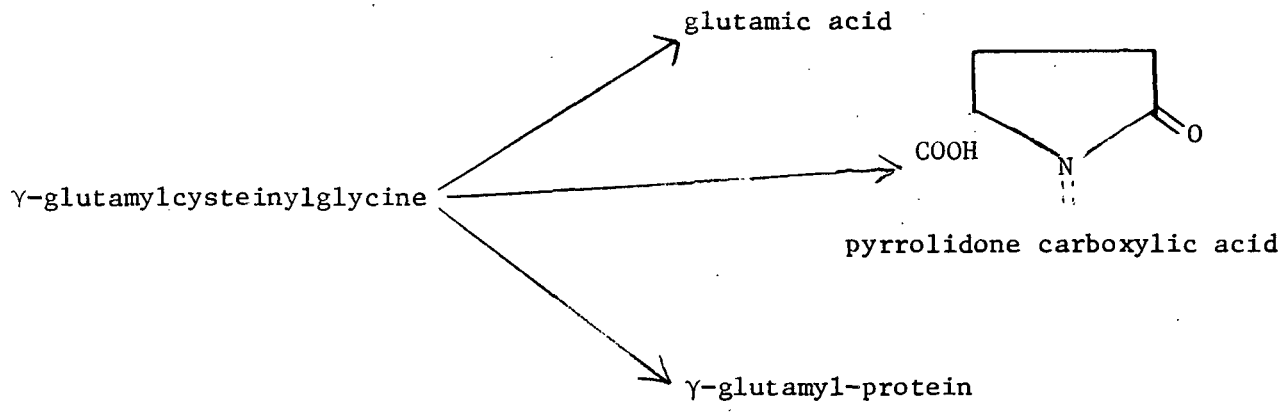
1) Glutamic acid + cysteine $\xrightarrow[Mg^{+2}]{ATP}$ γ-glutamylcysteine

2) γ-glutamylcysteine + glycine $\xrightarrow[Mg^{+2}]{ATP}$ γ-glutamylcysteinyl-glycine

Scheme IV. Biosynthesis of Glutathione

MODE OF DEGRADATION IN LIVING SYSTEMS

The catabolism of glutathione begins with the removal of the γ-glutamyl group followed by the hydrolysis of the cysteinylglycine to cysteine and glycine (85). The γ-glutamyl groups may be converted to glutamic acid, pyrrolidone carboxylic acid (86) or transfered to an amino acid of dipeptide. The hydrolysis and transfer reaction are catalyzed by γ-glutamyl-transpeptidase while the cyclization reaction is catalyzed by γ-glutamylcyclo-tranferase. The biodegradation of glutathione is shown in Scheme V.




Scheme V. Catabolism of Glutathione

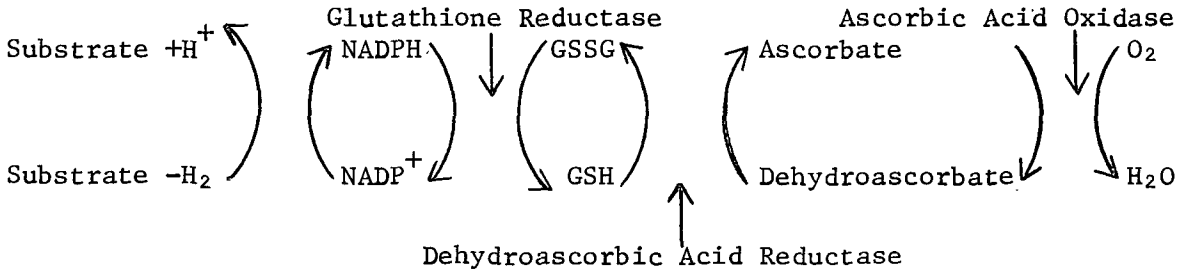
ENZYMES CONTROLLING OXIDATION-REDUCTION POTENTIAL

Some of the enzyme-catalyzed reactions in which thiols and disulfides participate do not contribute appreciably to their synthesis or degradation. The role of GSH and GSSG in controlling oxidation-reduction systems is one such important function. The enzymes which govern this redox potential involving GSH and GSSG will be described here.

From his knowledge of the properties of reduced glutathione and ascorbic acid, Szent-Gyorgyi in 1931 postulated a scheme whereby these substances might act as a respiratory chain (87). The subsequent discovery of dehydro-ascorbic acid reductase by Crook and Hopkins in 1938 (88) and of glutathione reductase by Mapson and Goddard in 1951 (89) made such a hydrogen transport chain tenable. Finally, in 1955, Mapson and Moustafa (90) showed that up to 25% of the total respiration in a pea seedling cotyledon may be passing over this route. The constituents of this alternate respiratory pathway and the enzymes controlling the redox states are shown in Scheme VI.

In light of this scheme the redox equilibrium position of the sulfhydryl pool in plants may be determined by the levels of glutathione reductase,

dehydroascorbic acid dehydrogenase, and ascorbic acid oxidase as well as the supply of NADPH.




Scheme VI. Alternate Respiratory Chain in Plants

ROLE IN CELL DIVISION

The biochemical interest in sulfhydryl groups was stimulated by Hopkins in 1921-28 with the discovery and isolation of glutathione (91). Shortly after this, in 1931 Rapkine (92) suggested the possible participation of thiols in cell division. Rapkine found that the free sulfhydryl concentration changed during cell division. He thus postulated that a cyclic oxidation-reduction process involving thiols and their oxidized forms took place during sea urchin egg mitosis. Mazia (93) showed that some soluble SH donor (presumably glutathione) engaged in a reversible reaction which first reduces the spindle protein precursor molecules to open up intramolecular SH sites, then oxidizes these sites to intermolecular SS linkages, thus forming the spindle. Anti-SH agents are known to inhibit cell division whereas the addition of glutathione or other SH compounds can initiate mitosis in a number of systems such as yeast cells and protozoa.

Glutathione is not the only form of free sulfhydryl involved in cell division. The presumed requirement for high sulfhydryl content is met differently according to cell type. Thus glutathione plays an essential role

in division in the lily microspore and pea seedling (94,95). An absolute increase in the concentration of this substance before and well after mitosis is observed. On the other hand the critical cell division sulfhydryl in the sea urchin egg, Chlorella, and yeast is a SH-containing protein or polypeptide (96).

In plants the embryonic tissue is characteristically in a reduced state. Thus as plant seeds begin to germinate their GSH level rises from nearly zero due to the reduction of GSSG (97). Evidence presented by Van Fleet (98) indicates that the young regions of plant shoots are characteristically in a reduced state which switches to an oxidized state as the tissue matures and differentiates. Further studies have shown that the SH gradient along the axis of higher plants is parallel to the IAA content (99) and correlates inversely with IAA oxidase activity (100). These findings have led Ram et al. (101) to suggest that plant growth regulators may act as regulatory substances which favor the juvenile state not only by preserving endogenous auxin levels but also by preserving SH levels by lowering peroxidase activity.

Spruce needles as well as the leaves of other evergreen plants show a periodic seasonal variation in glutathione and glutathione reductase with significantly increased levels in the winter (102). Levitt (103) has proposed that high glutathione levels are necessary to prevent oxidation of SH groups of protein and increase resistance to frost. Young needles have also shown high quantities of GSH, supporting the idea that the juvenile state is associated with the high SH content of the cells (102).

To summarize this section, the redox state of a cell is determined by components other than just sulfhydryl compounds. The levels of factors like NADH, NADPH, and oxygen tension, for example, can shift the potential to a

more reducing or oxidizing state. These additional cellular redox factors are interconnected, however, with the sulfhydryl pool. Thus the overall cellular redox state is considered to be reflected in glutathione levels, since glutathione is the major component of the sulfhydryl pool. High levels of reduced glutathione then would be indicative of a cell in a reducing state while low GSH levels or high GSSG would indicate an oxidizing state. While the SH-SS redox state is of importance (shifts to the left could lead to division, to the right to growth inhibition), it is now widely accepted that the existence of a single, causal mitotic substance is unlikely. Therefore, although studies have shown the SH compounds can in some cases induce cell division where nutritional effects can be excluded, the SH group is probably but one of the many factors in the "mitotic pool" of metabolism as viewed by Stern (104).

## ALBERT SZENT-GYORGYI'S "ELECTRONIC THEORY OF CANCER"

The theory (4) begins by dividing the history of life into two periods: the periods preceding and following the appearance of light and oxygen. In the presumed strongly reducing anaerobic atmosphere of the first period, the proteins must have consisted of soluble, closed-shell dielectric molecules, with paired electrons and saturated energy bands. In order to ensure their existence, these simple life forms had to proliferate as fast as conditions would permit. The strongly reducing atmosphere, therefore, must have contained chiefly electron donors and lacked electron acceptors. Among the donating groups, the strongly reducing SH must have played an important role in the process of proliferation.

The main functions of living systems in this stage of biological organization must have been fermentation and proliferation, neither of which demands

structure or specific electronic qualities. Proliferation was thus favored by the absence of structures and the simplicity of design. Reactions had to be molecular, molecules and ions being the smallest particles capable of independent motion. It is this stage of development that Szent-Gyorgyi terms the reducing "α state".

The situation changed drastically, however, with the appearance of oxygen. Being a strong electron acceptor, oxygen could separate electron pairs, transforming molecules into free radicals and desaturating energy bands making them conductant. This lent a high reactivity and mobility to electrons. The result was to desaturate energy bands forming semiconductors out of what were once dielectrics. The resulting creation of unbalanced forces could link protein molecules together forming increasingly complex structures leading to differentiation. This new oxidizing state of organization is referred to by Szent-Gyorgyi as the "β state" to distinguish it from the fermentative α state.

Also a brake had to be put on cell proliferation which was disfavored anyway by the cohesive forces of a more solid state and bulky oxidative machinery. Since cell division involves a complete rearrangement of the cellular interior, it is possible only in a semiliquid state. Therefore, cells containing extensive semisolid structures cannot divide. To be able to divide, these cells have to dedifferentiate, dissolve part of their structures, and return to an extent to the proliferative α state. After completing division they have to find their way back to the β state and rebuild their structures. Thus the α-β state transformation is reversible but must be supervised by a complex regulatory system.

How could oxygen then have induced all these changes which accompanied the transition from the fermentative and proliferating α state to the organized, later β state? Could oxygen, which is the general electron acceptor of the biosphere, be inducing conductivity in proteins by taking electrons from it? This can't be, for oxygen does not desaturate protein energy bands. The possibility exists, however, that some product of oxidation might contain oxygen in a form capable of acting as a single electron acceptor. The dicarbonyl with its extended pi electronic system is just such an arrangement. An example of this class of compounds is methylglyoxal. Since every peptide bond in a protein contains nitrogen with nonbonded electrons, these might easily be given off. The question then was whether methylglyoxal with an empty orbital in the pi electron system could accept an electron in a charge transfer reaction from an amino N of proteins in the ground state. Evidence that this indeed occurs was obtained through ESR spectrometry which showed the presence of a free radical upon mixing an aromatic amine with glyoxal and acidifying (105). The addition of methylglyoxal to an aliphatic amine did not give a free radical. When repeated, however, in the presence of physiological concentrations of glutathione (0.1%), a free radical spectrum was obtained. Further evidence for the possibility of a charge-transfer reaction between methylglyoxal and structural proteins was supplied by molecular orbital calculations (106) and the presence of methylglyoxal in an insoluble fraction from liver (76). This is consistent with biological systems also because all cells contain an enzyme system capable of inactivating the glyoxals, the glyoxalase system.

According to the theory, methylglyoxal or some other dicarbonyl acts as a cell division brake keeping the cell in the resting state. This involves maintenance of semiconductivity in structural proteins as well as the binding

of sulfhydryl groups vital to cell division. The glyoxalase system must be kept separated from the methylglyoxal by being enclosed in a vesicle or bound to a membrane. When cell division is required the glyoxalase is released and metabolizes the methylglyoxal brake allowing cell division to proceed.

## THESIS OBJECTIVES AND APPROACH

The ideas of Szent-Gyorgyi in his cell division hypothesis form the basis for this research. It must be emphasized, however, that this thesis did not attempt to prove or disprove the hypothesis. This thesis merely tried to discern the possible role of glyoxalase and methylglyoxal in Douglas-fir needle and needle callus growth and development. This thesis examined the metabolism of methylglyoxal in Douglas-fir needle tissue and Douglas-fir needle callus as representatives of resting (organized) and proliferative (unorganized) states respectively. Key questions to be answered with respect to each of these states are:

a) what is the level of glyoxalase activity in each of these states?
b) are there any alternate methylglyoxal catabolic routes present, for example, methylglyoxal reductase?
c) is methylglyoxal synthetase present?
d) can methyglyoxal be isolated from an insoluble fraction from either source and, if so, how much?
e) is glutathione present in the needle callus and, if so, does it fluctuate with number of subcultures or during a subculture period?
f) are the enzymes which regulate the redox state of glutathione present in each tissue type?
g) how do the properties of glyoxalase I from needle callus compare to those in the literature?

As a reference system, tobacco sources were also studied for their enzyme complement. It was anticipated that the answers to the above questions would allow enough insight on the metabolism of methylglyoxal in Douglas-fir needle and needle callus so that a role, if any, in growth and development might be proposed for this substance and glyoxalase.

# METHODS AND MATERIALS

## DOUGLAS-FIR SEEDLING NEEDLE SOURCE

Douglas-fir needles were obtained from seedlings grown under greenhouse conditions at The Institute of Paper Chemistry. The seedlings were started at the Institute from seeds obtained from the Weyerhauser Company seedlot 491-15-1 CH1002. This seedlot was collected at an elevation of 1500 feet from a point west of the Cascades near East Roseburg, Oregon.

Seed preparation involved soaking in tap water for 48 hours followed by cold stratification at 1-2°C for between 60 to 90 days. The seeds were then placed in soil consisting of: 3 parts silted clay loam, 2 parts mason sand, 2 parts German peat, 1 part purolite, and 3 parts decomposed peat at a depth of 1-1/2 inches in redwood flats. Upon germination, the seedlings were watered as needed with tap water and fertilized once a month during the growing season with 2 teaspoons per gallon of 20:20:20 (nitrogen:phosphorus: potassium). The fungicide, Captan, can be added when microbial growth is observed. During the winter months, the seedlings were moved to a cooler portion of the greenhouse for a cold treatment. The temperature ranged from 6-7°C and the day length was 15 hours as provided by supplemental lighting. Summer daytime temperatures fluctuated between 21-32°C with the greenhouse windows set to open at 21°C. Douglas-fir seedlings grown in this manner are shown in Fig. 1.

## DOUGLAS-FIR NEEDLE CALLUS SOURCE

The initiation and perpetuation of Douglas-fir needle callus is well established at the Institute and has been going on for a number of years (107). The initiation process involves collecting seedling needles from the greenhouse

and treating them to remove and kill adsorbed microbes. The technique involves washing the needles with soap and water followed by a 50% Hi-lex solution for 30 minutes. The bleach is decanted and the needles are rinsed twice with sterile water. The now aseptic needles are then dissected into short segments approximately 1.0-1.5 cm long and placed in petri dishes. The dishes contain a modified Murashige and Skoog medium as described in Appendix I. The petri dishes are sealed with Parafilm and kept in incubators. Fluorescent lamps provided the lighting at from 125-175 footcandles. The day length was set at 16 hours with a temperature of 23°C while the 8-hour dark period temperature was 19°C.

Figure 1. Douglas-fir Seedlings

After six to eight weeks the needles have produced an unorganized mass of new cells or callus at the wounded site which may then be continually subcultured. Subculturing to fresh medium once a month allows the rapid buildup of callus material as well as being necessary for the survival of the cells. The callus initiation sequence is depicted in Fig.2

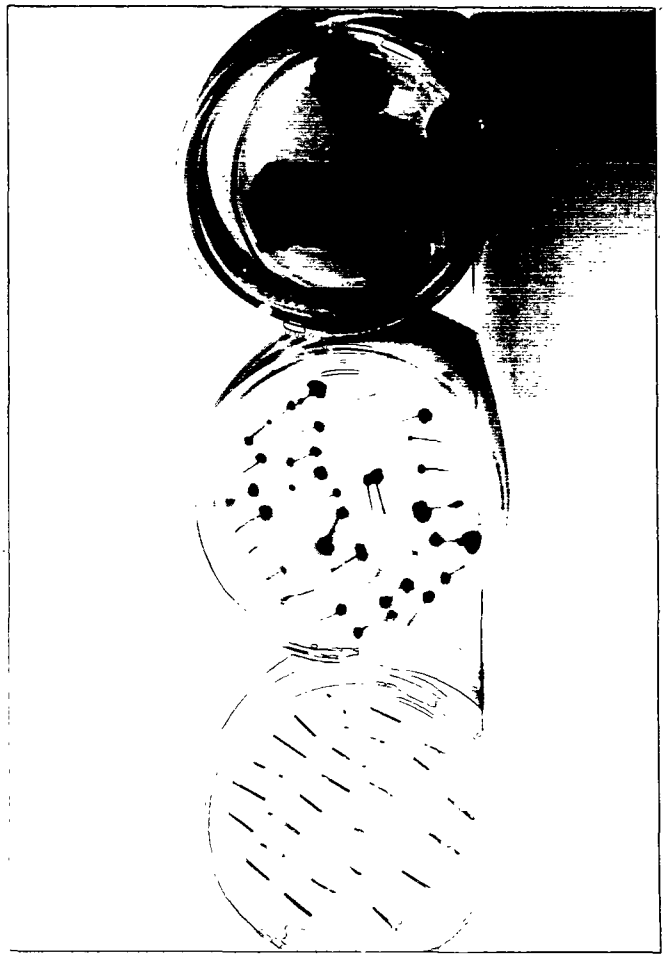
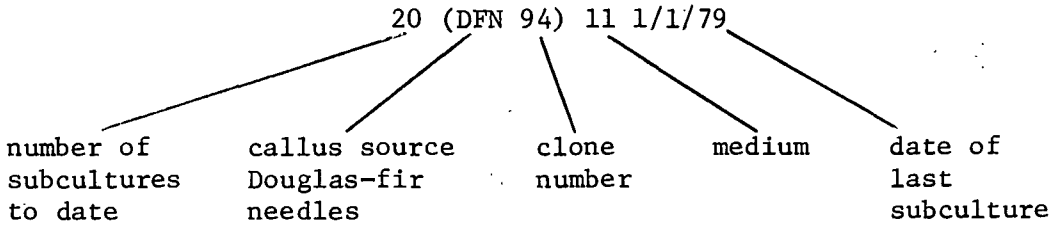


Figure 2. Callus Initiation

The history of various clones of callus material is recorded according to the following system.




TOBACCO CALLUS, CROWN GALL, AND SEEDLING SOURCES

All three of these tobacco (Nicotiana tobaccum) tissue types were gifts from Professor Folke Skoog of the University of Wisconsin Department of

Botany. The tobacco callus and crown gall were supplied as such while the third tissue type was in seed form, from which seedlings were grown.

The tobacco callus, isolated in March of 1976 and initiated from the pith region, was grown in the dark at 24°C on the modified Murashige and Skoog medium (Appendix I). The hormonal supplements however were varied in a three month cyclic fashion giving rise to the stages $T_1$, $T_2$, and $T_3$. During the first month or $T_1$ stage, the medium contained 20 ppm IAA and kinetin. The $T_2$ stage added gibberellic acid at 2 ppm $GA_3$ and dropped the kinetin to 3 ppm. The third month or $T_3$ stage eliminated the gibberellic acid. Because it was grown in the dark the tissue was white and also very hard, closely resembling the aspen callus previously cultured in this laboratory.

The tobacco crown gall tissue, also initiated from the pith region, was likewise grown in the dark at 24°C. This tissue was white and extremely friable. The crown gall tissue was divided into three sets based on the ability of the three different cell types to synthesize aberrant polyamines. The three crown gall sets B,C, and E were thus denoted by their ability to synthesize octopine, nopaline, and neither, respectively. This ability arises in the tissue as a result of being initiated by various strains of Agrobacterium tumefasciens. Since the crown gall represents a true plant tumor tissue it can be subcultured on medium without added hormones. All tobacco crown gall types were thus grown on the basic Murashige and Skoog medium without hormonal supplements. The tissue was subcultured once a month. Tobacco callus and crown gall tissues are shown in Fig. 3.

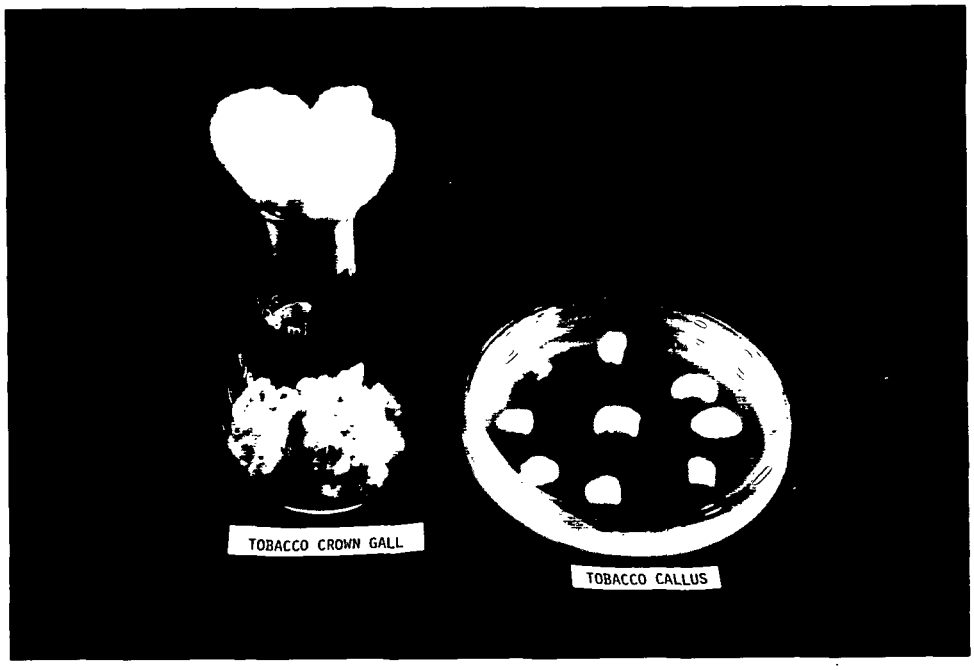



Figure 3. Tobacco Crown Gall and Callus Respectively

The tobacco seedlings were grown in the Institute greenhouse from the supplied Havanna 142 seeds. The seeds had been cold stratified previously and were grown under the same conditions as denoted for the Douglas-fir seedlings with the exception that clay pots were used in place of redwood flats. The tobacco seedlings are shown in Fig. 4.

## NONPROTEIN SULFHYDRYL ANALYSIS

### EXTRACTION PROCEDURE

Analysis of needle callus for nonprotein sulfhydryl content was by means of acetone powders. The tissue was ground in cold acetone and centrifuged as previously described. The acetone soluble fraction was evaporated in a water bath at 50°C with a nitrogen stream until no further acetone remained. The residue, which contained some cellular water, was diluted to 5.0 cc with potassium phosphate buffer at pH 6.8 at an ionic strength of 0.05 I. This

suspension was well mixed with a Vortex mixer and extracted twice with equal volumes of ethyl ether. The remaining aqueous phase was assayed for non-protein SH.

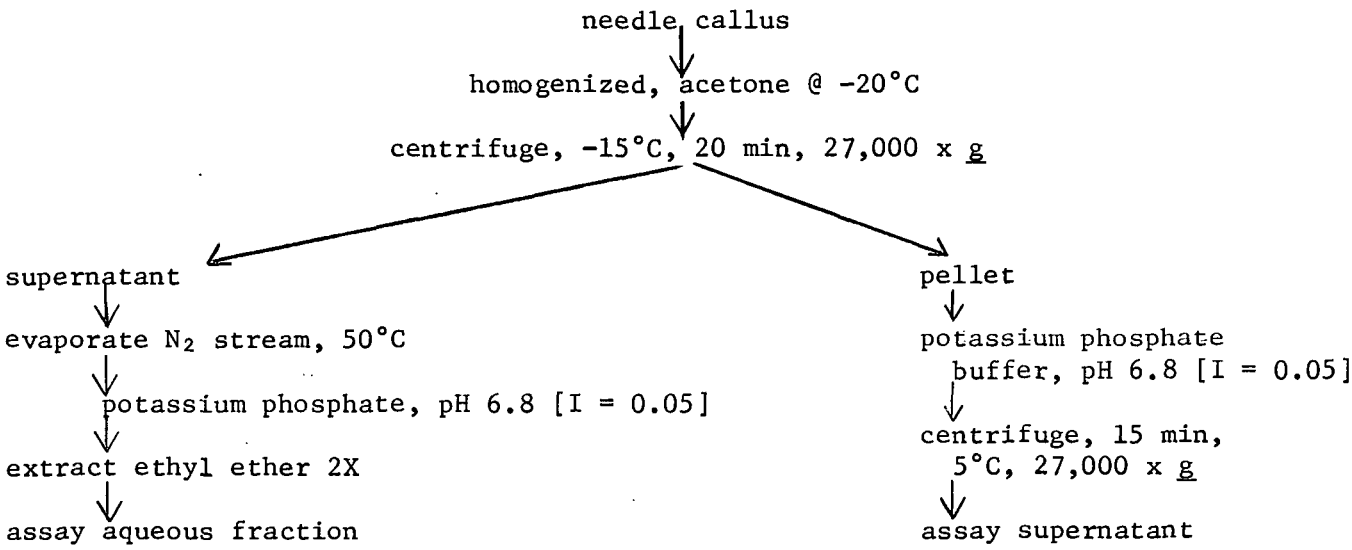
Figure 4. Tobacco Seedlings

The pellet remaining from the acetone powder was also extracted with 10.0 cc of 0.05 I phosphate buffer pH 6.8 for one hour under continuous stirring at 1-2°C. This solution was then centrifuged at 27,000 x g for 15 minutes at 5°C and the resulting supernatant assayed for nonprotein SH. The extraction procedure is shown in Scheme VII.

SPECTROPHOTOMETRIC ASSAY

In 1959 Ellman (108) was the first to use di-(5-carboxy-4-nitrophenyl) disulfide (DTNB) at pH 8 to estimate sulfhydryl groups. This disulfide is reduced in the presence of nonprotein SH to the corresponding aromatic thiol compound. This substance, unlike the colorless disulfide, has an intense yellow color which can be used to estimate its concentration. It was

later found by Jocelyn (109) that, if the pH was reduced to 6.8, only non-protein sulfhydryls such as GSH and cysteine react with DTNB. This technique was used throughout to determine nonprotein sulfhydryl content of tissue extracts.




Scheme VII. Extraction Procedure for Nonprotein SH

A calibration curve was constructed by plotting the concentration of GSH vs. the absorbance at 412 nm. The sample cuvette contained 49.8 µmoles of potassium phosphate buffer pH 6.8, 0.5 µmoles DTNB, and GSH at 0.325, 0.244, 0.163, 0.081, 0.016, and 0.003 µmoles. The reference cuvette contained 49.8 µmoles of potassium phosphate buffer pH 6.8 and 0.5 µmoles of DTNB. Both cuvettes had a final volume of 3.0 cc. The absorbance was read 5 minutes after the addition of the sample. The calibration curve is shown in Appendix III.

For tissue samples, 0.50 cc of extract was added to the sample cell in place of the standard GSH solutions. The buffer and DTNB levels were the same as for the calibration system. Blanks were run where the DTNB was substituted with just buffer. These readings were subtracted from the values

with DTNB, before estimating the nonprotein sulfhydryl content. The DTNB was obtained from Sigma Chemical Co.

SEPARATION OF GLUTATHIONE AND CYSTEINE USING DEAE SEPHADEX G-25

The resolution of a standard solution of glutathione and cysteine was carried out on an ion exchange column according to the procedure of Esterbauer and Grill (110). A sample containing 1.0 mg of GSH and 1.0 mg of cysteine was dissolved in 2.0 cc of 40 mM sodium acetate buffer at pH 4.0. This sample was applied to a 1.3 x 25 cm DEAE Sephadex G-25 column equilibrated with the above buffer. The sample was eluted at a flow rate of 60 cc per hour and fractions were collected at 3.0 cc intervals. A linear NaCl gradient from 0.0 to 0.6M was added to remove the bound glutathione. Eluting sulfhydryl compounds were detected with DTNB. The DEAE Sephadex G-25 was purchased from Sigma Chemical Co.

IDENTIFICATION OF GLUTATHIONE IN A NEEDLE CALLUS EXTRACT BY AMINO ACID ANALYSIS

The adjacent sulfhydryl positive fractions collected from the DEAE Sephadex column were pooled and freeze-dried. The resultant white powder containing 202 µg of sulfhydryl material was dissolved in 0.6 cc of double distilled $H_2O$ (dd $H_2O$) and loaded on a Bio-Gel P-2 column. The column was 1.3 x 16.0 cm and was eluted with dd $H_2O$ at 24 cc/hour. The eluate was monitored at 254 nm at a range of 0.1A. Again the DTNB positive fractions were pooled and freeze-dried giving a white powder. A portion of this sample was dissolved in dd $H_2O$ and the UV spectrum taken. The spectrum was very similar to reduced glutathione. The remainder of the sample was oxidized with performic acid by the procedure of Giovanelli et al. (111). The procedure involved reacting 9.0 cc of 88% formic acid with 1.0 cc of 30% $H_2O_2$ for one

hour at 27°C. This solution was added to the sample for 30 minutes at 27°C and then evaporated under a nitrogen stream at 80°C to dryness. The resultant material was dissolved in 0.5 cc of 0.2M lithium citrate buffer, pH 2.83, and loaded on a Beckman Amino Acid Analyzer Model 119CL. After one bed volume of buffer, the next 1.0 cc fraction was collected and saved. This solution was placed in a hydrolysis tube with 1.0 cc of constant boiling HCl (approx. 5.7N) and purged with nitrogen. The tube was placed in a dry ice-acetone bath, vacuum evacuated and purged with nitrogen three times. The tube was sealed and kept at 110°C for 22 hours after which it was dried over NaOH under vacuum at 60°C. After dissolving in lithium citrate buffer as before, the complete amino acid analysis was done using ninhydrin and three buffer changes. The lithium citrate buffers were 0.2M pH 2.83, 0.2M pH 3.70, and 1.0M pH 3.75 and were prepared according to Beckman specifications. The Bio-Gel P-2 was a product of Bio-Rad.

## EXTRACTION AND IDENTIFICATION OF METHYLGLYOXAL FROM MATURE DOUGLAS-FIR NEEDLES

The isolation and identification of methylglyoxal from Douglas-fir needles was by means of its 2,4-dinitrophenylosazone. The technique employed was a slight modification of the procedure of Fodor et al. (76), who isolated methylglyoxal from beef liver. Advantage was taken of the possibility that if methylglyoxal was present in Douglas-fir needles it should be bound to structural protein. As such it would be found in an insoluble fraction, free from a host of other compounds.

Mature needles (900 g) were collected from 56 week old Douglas-fir seedlings from seedlot 491-15-1 CH 1002. The needles were homogenized three times in a blender in cold acetone at -20°C and vacuum filtered on a Buchner

funnel with Whatman no. 1 filter paper. The resulting white powder was spread out on a tray and allowed to air dry overnight to remove any residual acetone. The powder was then homogenized in 1500 cc of dd $H_2O$, the pH adjusted to 4.5, and again homogenized. This suspension was centrifuged for 10 minutes at 10,000 x g. The supernatant containing soluble proteins was discarded. The residue meanwhile was suspended in 1.0 liter of 1N HCl and 5.0 g of 2,4-dinitrophenylhydrazine was added under strong stirring. This mixture was allowed to stir for 6 days at room temperature and was then homogenized in 1.0 liter of ethyl acetate. The suspension was centrifuged for 15 minutes at 10,000 x g. The ethyl acetate layer was then washed with dd $H_2O$ and dried over anhydrous $Na_2SO_4$. After gravity filtration the ethyl acetate was removed with a rotary evaporator. The remaining residue was washed 8 times with 50.0 cc portions of 60-90°C petroleum ether and then extracted 6 times with 50.0 cc portions of benzene. The benzene extract was gravity filtered and the benzene removed with a rotary evaporator leaving a 600 mg residue. This residue was chromatographed on a 1,000 micron thick Silicagel GF plate with toluene:60-90°C petroleum ether:ethyl acetate (34:5:7). The colored band corresponding in $R_f$ to the known methylglyoxal osazone was removed from the plate and eluted from the silica gel with chloroform. This material was chromatographed in benzene:chloroform (2:1), the corresponding band again removed, and rechromatographed a final time in the original solvent system. The band at the proper $R_f$ was then removed and characterized. The UV-VIS spectrum in chloroform, the VIS spectrum in 10% KOH in 80% MeOH, the IR spectrum, and melting point were determined.

Due to the nature of the sample that was derivatized, possible interferences from lignin, cellulose, and hemicelluloses were tested. One gram samples of p-dioxane lignin, cellulose, xylan, and arabino-galactan were treated with

10.0 cc of 2,4-dinitrophenylhydrazine in 1N HCl for 30 minutes at 80°C. The solutions, upon cooling, were extracted with ethyl acetate and the spectra determined in 10% KOH in 80% MeOH.

The 2,4-dinitrophenylhydrazine and benzene were purchased from Matheson, Coleman, and Bell. The ethyl acetate and toluene were products of Mallinckrodt, Inc. The cellulose was powdered chromatographic media from Whatman, Inc. Xylan and arabino-galactan were gifts from Dr. Norman Thompson of the Institute staff. The p-dioxane lignin was isolated by Thomas Crozier as part of his Ph.D. thesis here at the Institute. All UV-VIS and VIS spectra were run on the Perkin-Elmer Model 576 recording spectrophotometer. The IR spectrum was determined on a Perkin-Elmer 621 grating infrared spectrophotometer. The melting point was run on a Thomas Hoover capillary apparatus.

900 grams needle
|
homogenized in cold acetone
|
vacuum filtered, air dried
|
powder homogenized in 1500 cc dd $H_2O$
|
pH adjusted to 4.5, homogenized
|
centrifuged 10 min, 10,000 x g
|
suspend residue in 1 liter 1N HCl
|
add 5.0 grams 2,4-DNPH
|
stir 6 days room temp.
|
homogenized 1 liter ethyl acetate
|
centrifuged 10,000 x g, 10 min
|
ethyl acetate washed with dd $H_2O$, dried with $Na_2SO_4$
|
gravity filtered
|
evaporated under reduced pressure
|
residue washed with 400 cc 60-90°C pet. ether
|
residue extracted with 300 cc benzene
|
benzene extract gravity filtered
|
evaporated under reduced pressure
|
residue (600 mg) suspended in chloroform
|
chromatographed on Silicagel GF plate
toluene:pet.ether:ethyl acetate (34:5:7)
|
chromatographed on Silicagel GF plate
benzene:chloroform (2:1)
|
chromatographed on Silicagel GF plate
toluene:pet.ether:ethyl acetate
|
characterized

Scheme VIII. Extraction and Identification of Methylglyoxal Osazone

## DOUGLAS-FIR SUSPENSION CELL GROWTH CURVES

The basal medium for this study was as indicated in Appendix I with napthalene acetic acid (NAA) at 1 ppm as the only hormone. Seven liters of the medium minus NAA were prepared, pH adjusted to 5.8 with KOH, and autoclaved. The NAA was then added to the level of 1 ppm by means of filter sterilization. The tissue 11(DFN 104)2 was removed from plates and placed in 125 cc flasks containing 40 cc of medium. Callus from a total of 71 plates was dispersed among 24 flasks. The flasks were placed on a shaker for one hour at 120 rpm, swirled, the large chunks allowed to settle, and then filtered through 500 micron screens. The screened material was pooled and diluted to 1400 cc with medium. This suspension served as the cellular stock solution used to inoculate the nipple flasks. The experiment involved fourteen nipple flasks which allowed two flasks for each of the six treatments plus two for the control. These were prepared by diluting 200 cc of the stock solution to 900 cc with medium. This allowed an initial 70 cc for the zero time analysis plus 415 cc for each of the two nipple flasks per treatment. The six treatments consisted of adding $10^{-4}$, $10^{-6}$, and $10^{-8}$ M each of methyglyoxal and glutathione as an insignificant volume, after filter sterilization, to the 900 cc and the nipple flasks inoculated. The flasks were placed on drums rotating at 1 rpm under continuous lighting at 23°C $\pm$ 1°C. Samples were taken every other day during the course of the experiment.

Dry weight determinations were made as follows. Whatman No. 1 filter discs of 9.0 cm diameter were numbered and placed in an oven for 8 hours at 105°C. After desiccation the filters were tared to the nearest 0.1 mg. After folding the filters to assume the conical shape, they were seated in the glass filter with distilled water. A 10.00 cc suspension sample was drawn,

allowed to gravity filter, and then placed under vacuum by an aspirator for 60 seconds at 7.5 liters per minute of water flow. When all the samples had been collected in this manner, the papers were dried again at 105°C for 8 hours, desiccated, and weighed a second time giving the dry weights. Triplicate samples were withdrawn from each treatment and the control for every test date.

## ACETONE POWDERS

All tissues, both Douglas-fir and tobacco, were prepared for enzyme assays by making acetone powders. The callus tissues as well as the crown gall material were all homogenized by hand in a Ten Broeck grinder. The acetone as well as the homogenizer and centrifuge tubes were kept in a deep freeze at -20°C. The tissues were homogenized in the cold acetone (5:1, v/w) in the deep freeze rapidly but thoroughly. After homogenization the suspension was centrifuged in a Sorvall RC2-B at 27,000 x g for 15 minutes at -15°C. The acetone phase, containing pigments such as chlorophyll and fatty acids, etc., was decanted and discarded. The remaining pellet containing all the cold acetone insoluble substances such as protein and carbohydrate could then be extracted with the appropriate buffer giving an aqueous enzyme solution.

The buffer extraction procedure involved adding the desired volume and type of buffer directly to the pellet in the centrifuge tube. A glass rod was used to disperse the pellet in the buffer. A magnetic stirring bar was added to the tube and the contents stirred continuously in the cold at 2-3°C for one hour. Following centrifugation at 5°C, 27,000 x g for 20 minutes, the supernatant was decanted and used directly in the enzyme assays.

The Douglas-fir needles, being more durable than the callus, were first subjected to cold acetone in a Virtis homogenizer at 0.5 amps for one minute. This suspension was centrifuged as before and the pellet was extracted with cold acetone a second time with the hand homogenizer. Following a second centrifugation, the resultant pellet could then be buffer extracted for its enzyme complement as already described. This two step homogenization procedure was found to work very well for the needle samples.

## ENZYME UNITS

All enzyme units are expressed as nmoles of product produced or substrate decomposed per minute at 25°C. The molar absorptivity coefficient of S-lactoylglutathione used for the glyoxalase enzyme system was 3370$\underline{M}^{-1}$ $cm^{-1}$ at 240 nm (81). Those enzymes requiring either of the two nicotinamide containing coenzymes were assayed at 340 nm using 6220$\underline{M}^{-1}$ $cm^{-1}$ as the molar absorptivity coefficient. A sample enzyme unit calculation is shown in Appendix II.

## ENZYME ASSAYS

A Perkin-Elmer 576 ST recording spectrophotometer was used for making all enzyme activity measurements. Unless otherwise stated, all solutions were prepared or diluted with dd $H_2O$. All enzymes assayed were run with controls which showed no activity in the absence of added enzyme.

### GLYOXALASE I

Glyoxalase I was assayed by a modification of the method of Racker (21). The reaction was measured by following the increase in absorbance with time at 240 nm due to the formation of the thiol ester bond in S-lactoylglutathione. The reaction was run in 1.0 cm quartz cuvettes thermostated at 25°C. The

sample cuvette contained 3.3 μmoles of GSH, 17.8 μmoles of methylglyoxal, 30.0 μmoles of $MgCl_2$, 125.0 μmoles of potassium phosphate buffer at pH 6.8, and 0.10 cc of enzyme extract in a total volume of 3.0 cc. The reference cuvette meanwhile contained the same constituents except for the enzyme extract in a total volume of 3.0 cc.

All reactants, except the enzyme extract, were mixed together and allowed to react for 30 minutes. This gave the methylglyoxal and glutathione sufficient time to react nonenzymically. The cells were then placed in the spectrophotometer and the recorder pen and instrument zeroed. The reaction was initiated by adding 0.10 cc of enzyme extract to the sample cell, stirring with a glass rod, and starting the recorder pen trace. The rate of reaction was determined by the slope of the initial linear two minute portion of the plot.

Methylglyoxal was obtained from Sigma Chemical Co. as a 40% aqueous solution which was diluted and used as a 2.5% solution. Reduced glutathione was also obtained from Sigma in powder form and prepared as a 2.0% solution. The magnesium chloride as well as the mono- and dibasic potassium hydrogen phosphate were products of Mallinckrodt, Inc. The pH 6.8 phosphate buffer was prepared according to the procedure of Sorensen (112).

GLYOXALASE II

Glyoxalase II assays were carried out by a modified Racker method (21). Because of the inhibitory effect of phosphate ions on glyoxalase II activity, the acetone powders were extracted in 50 mM Tris-HCl, pH 7.4, instead of phosphate buffer. The activity was followed spectrophotometrically at 240 nm by following the decrease in absorbance of the thiol ester intermediate with time. The sample cuvette contained 137.5 μmoles of Tris-HCl, pH 7.4, 1210 μmoles

of S-lactoylglutathione, and 0.20 cc of enzyme extract in a total volume of 3.0 cc. The reference cuvette contained 147.5 µmoles of Tris-HCl, pH 7.4, and 1210 µmoles of S-lactoylglutathione in a total volume of 3.0 cc. The cuvettes were zeroed and the reaction initiated with the enzyme extract. The Tris buffer and the S-lactoylglutathione, supplied as the barium salt, were products of Sigma Chemical Co.

METHYLGLYOXAL REDUCTASE

Methylglyoxal reductase activity was measured by a modified procedure of Willetts and Turner (66). The reaction was monitored by measuring extinction changes at 340 nm. The reduced forms of the nicotinamide coenzymes give a decrease in absorbance at 340 nm upon oxidation. Both the sample and the reference cells contained 115 µmoles of potassium phosphate buffer, pH 6.8, 2.5 µmoles of NADPH or NADH, and 0.20 cc of enzyme extract. The reaction was initiated by the addition of 35.6 µmoles of methylglyoxal to the sample cell. The total volume in each cuvette was 3.0 cc. The coenzymes, NADPH and NADH, were purchased from P-L Biochemicals.

DL-LACTALDEHYDE DEHYDROGENASE

The $NAD^+$ linked DL-lactaldehyde dehydrogenase converts the lactaldehyde produced by methlyglyoxal reductase activity to DL-lactate (66). Since DL-lactaldehyde could not be obtained commercially, it was synthesized from DL-threonine according to the procedure of Huff and Rudney (113). A 1.0% solution of threonine was heated at 100°C for 4 minutes in equimolar ninhydrin in order to deaminate the amino acid. The solution was allowed to cool to room temperature and 2.0 g of sodium bicarbonate were added per gram of threonine. The resultant brown precipitate was removed by vacuum filtration

on Whatman No. 1 paper. To the filtrate was added 1.0 g of Darco G-60 activated charcoal per 0.20 g of threonine and the suspension was centrifuged for 10 minutes at 14,000 x g. The resultant clear solution was treated with Dowex 50W x 8 (hydrogen form) and decanted, giving a 17% DL-lactaldehyde solution.

The enzyme assay began after extracting the acetone powders with 50mM Tris-HCl, pH 8.5. The resultant supernatant after centrifugation was assayed for DL-lactaldehyde dehydrogenase activity by the method of Huff and Rudney (113). Both the sample and the reference cells contained 37.8 μmoles of glycine-NaOH buffer at pH 10.3, 1.5 μmoles of $NAD^+$, and 0.20 cc of enzyme extract in a total volume of 3.0 cc. The reaction was initiated by the addition of 138.3 μmoles of DL-lactaldehyde and measured as the increase in absorbance at 340 nm. Glycine and DL-threonine were purchased from Sigma Chemical Co.

α-KETOALDEHYDE DEHYDROGENASE

The enzymic oxidation of methylglyoxal directly to pyruvate was measured as the initial increase in absorbance at 340 nm by the method of Monder (71). The standard assay system contained 2.5 μmoles of $NAD^+$ or $NADP^+$, 115 μmoles of Tris-HCl buffer, pH 8.5, and 0.20 cc of enzyme extract. The reaction was initiated with 71.2 μmoles of methylglyoxal in the sample cell. Final volume in each cuvette was 3.0 cc. The oxidized coenzymes were purchased from P-L Biochemicals.

METHYLGLYOXAL SYNTHETASE

The glyoxalase I coupled reaction of Hopper and Cooper (63) was used to assay methylglyoxal synthetase activity. Enzyme activity produces methylglyoxal which can then be acted upon by glyoxalase I and monitored at 240 nm.

Thus both the reference and the sample cell contained 1.0 I.U. of glyoxalase I, 0.20 cc of enzyme extract, 3.3 µmoles of GSH and 132 µmoles of Tris-HCl buffer, pH 7.4. The addition of 2.9 µmoles of dihydroxyacetone phosphate to the sample cell was used to initiate the reaction. The final volume in each cuvette was 3.0 cc. Glyoxalase I, extracted from yeast, was purchased from Sigma Chemical Co. suspended in an aqueous buffer. Dihydroxyacetone phosphate was supplied as the diketal also by Sigma Chemical Co.

GLUTATHIONE REDUCTASE

Glutathione reductase converts GSSG back to the reduced form, GSH. The procedure used to assay its activity was that of Esterbauer and Grill (102). The activity was measured spectrophotometrically by following the decrease in absorbance of NADPH at 340 nm. The sample and reference cells each contained 1.6 µmoles of NADPH, 10.5 µmoles of EDTA, 0.33 cc of 1.0% bovine serum albumin, and 1.0 cc of enzyme extract. The reaction was initiated by adding 10 µmoles of GSSG to the sample cell. Total volume in sample and reference cells was 2.25 cc. Bovine serum albumin was purchased from Miles Laboratories and the EDTA from Baker Chemicals.

## ISOELECTRIC FOCUSING

Isoelectric focusing was carried out on Ampholine PAGplate (LKB) gels. Samples of Douglas-fir callus and needle extract were run on prefocused gels with a pH gradient ranging from 3.5 to 9.5. Prefocusing for one-half hour allowed the gradient to be established before the sample was added. The anode solution consisted of 1M $H_3PO_4$ and the cathode solution was 1M NaOH. Aqueous protein samples were applied to the surface of the gels with 1.0 cm filter paper wicks. A constant power of 25 watts was applied for half an hour at which

time the filter paper wicks were removed. The same constant power was then reapplied for an additional 1-1/2 hours. The heat generated by the resistance due to the gel was dissipated with a water jacketed cooling block. Upon completion of the focusing period, the gel was stained either for protein or selectively for enzyme activity. Glyoxalase I activity was determined on the gel using the specific stain MTT tetrazolium according to the procedure of Aronsson et al. (36). The MTT tetrazolium was purchased from Sigma. The isoelectric focusing gels and apparatus were products of LKB Instruments, Inc.

## ULTRACENTRIFUGATION

Analytical ultracentrifugation was used to measure diffusion and sedimentation coefficients of Douglas-fir needle callus glyoxalase I. All determinations were made with a Beckman Model E Ultracentrifuge.

Determination of sedimentation velocity was done with a single sector, synthetic boundary type of cell. The sample boundary was monitored with Schlieren optics using Schlieren window holders and quartz windows. An Analytical-D type rotor was used and carefully counter-balanced. The solvent consisted of 0.05M potassium phosphate at pH 6.8 containing 0.20M NaCl. The rotor was run at 56,000 rpm and a temperature of 25°C. Photographs were taken at 2 or 4 minute intervals with 9 photos being taken over a period of 24 minutes.

The principle of the method is to subject solute molecules to high centrifugal forces and then measure their rates of sedimentation. The rate of the average of all the molecules is measured by recording the position of the boundary in the centrifuge cell as a function of time. The maximum

ordinate of the peak is a convenient and a sufficiently accurate indication of the boundary position. The slope of the plot of log x versus the time is used to calculate the sedimentation coefficient.

Diffusion coefficients were determined in a double sector synthetic boundary cell. For this determination interference optics were employed with interference window holders and quartz windows. Again an Analytical-D type rotor was used. The solvent was the same as for the sedimentation velocity determination. The rotor was run at 6,000 rpm at a temperature of 25°C. A total of five photographs were taken at two minute intervals.

The principle of the method is to subject the solute to low rotor speeds and form a sharp boundary in the cell. The diffusion coefficient is then determined from the spreading of the boundary with time. The diffusion coefficient is obtained by extrapolating a plot of the apparent diffusion coefficients versus time to zero time. Knowledge of the diffusion and sedimentation coefficients allows the calculation of the molecular weight, assuming a partial specific volume for a globular enzyme.

# RESULTS AND DISCUSSION

## IDENTIFICATION OF NONPROTEIN SULFHYDRYL COMPONENT IN DOUGLAS-FIR NEEDLE CALLUS

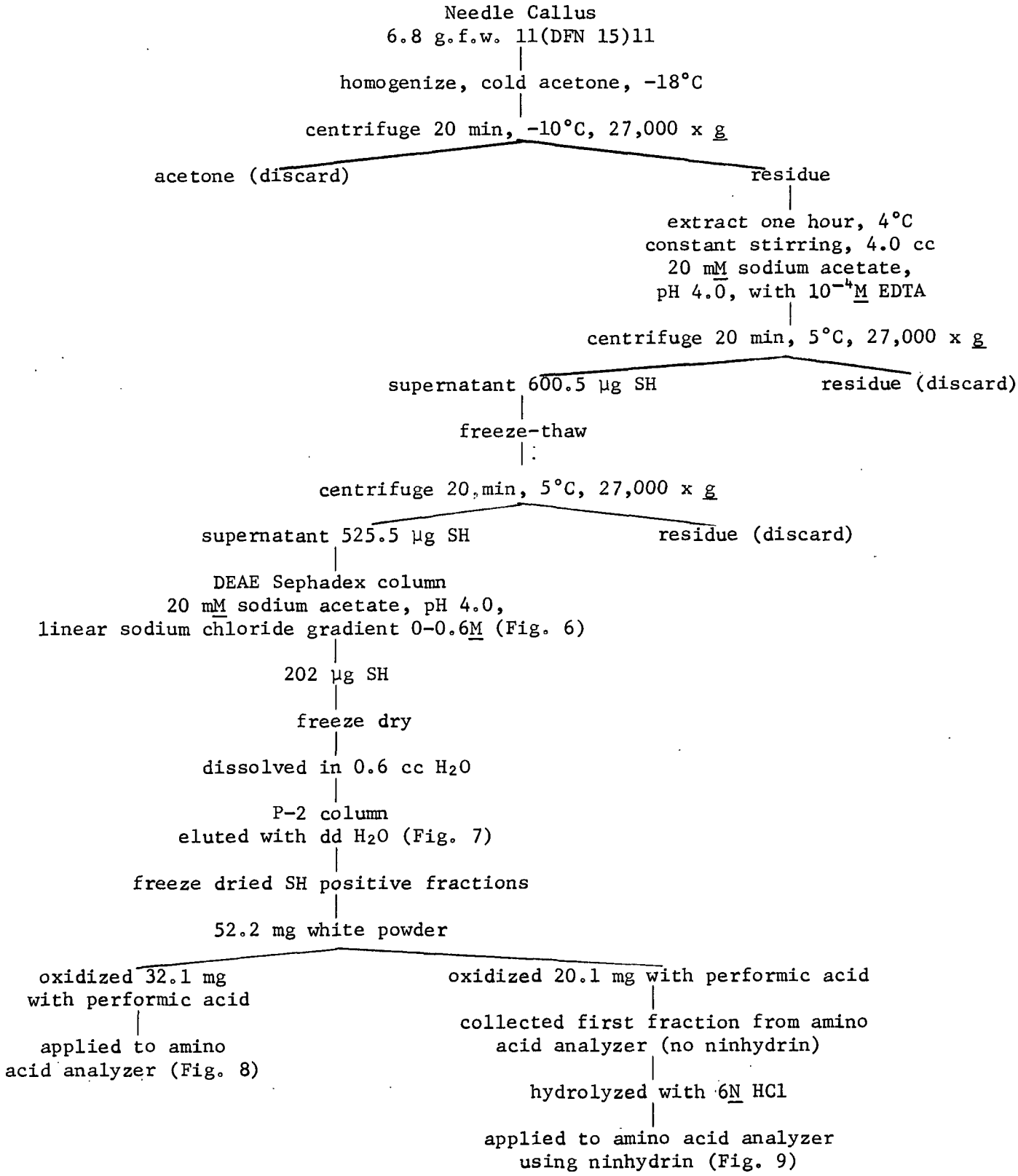
### ELUTION PATTERN ON DEAE SEPHADEX G-25

The identification of the nonprotein sulfhydryl component of Douglas-fir needle callus began by making an acetone powder from 6.81 g of 11 (DFN 15)11 (Scheme IX). The powder was extracted for one hour at 4°C in 4.0 cc of 20 mM Na acetate buffer at pH 4.0 with 0.1 mM EDTA. After centrifugation the supernatant was assayed for SH using DTNB and gave a value of 600.5 µg SH. This solution was freeze-thawed and centrifuged at 27,000 x g at 5°C for 20 minutes. The resulting supernatant was applied to a DEAE Sephadex G-25 column and eluted with a linear 0.0 to 0.6M NaCl gradient in 20 mM Na acetate, pH 4.0. A standard solution containing a mixture of cysteine and glutathione was likewise applied to the same column and eluted in a similar manner. The elution patterns are shown in Fig. 5 and 6.

Cysteine did not bind to the column as its elution volume is the same as one bed volume, about 23.0 cc. The glutathione meanwhile was removed only after the start of the NaCl gradient and had an elution volume of 46 cc. The sulfhydryl positive material from the needle callus, meanwhile, had an elution volume of 50 cc and at this point appeared to be glutathione.

### ELUTION PATTERN ON BIO-GEL P-2

The sulfhydryl positive fractions from the needle callus sample were then pooled and freeze-dried giving a white powder. The powder was dissolved in dd $H_2O$ and loaded onto a P-2 column equilibrated with dd $H_2O$. The sample was




Scheme IX. Extraction and Identification Procedure for Needle Callus Nonprotein Sulfhydryl Component

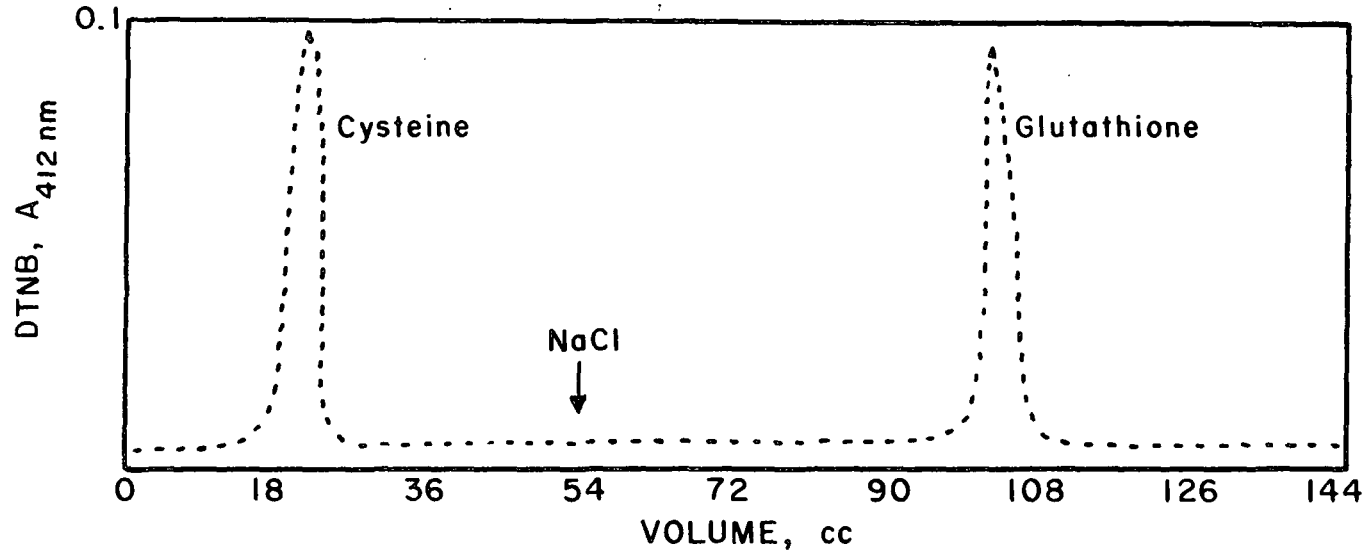



Figure 5. Elution Pattern for Standard Solution of Cysteine and Glutathione on DEAE Sephadex G-25

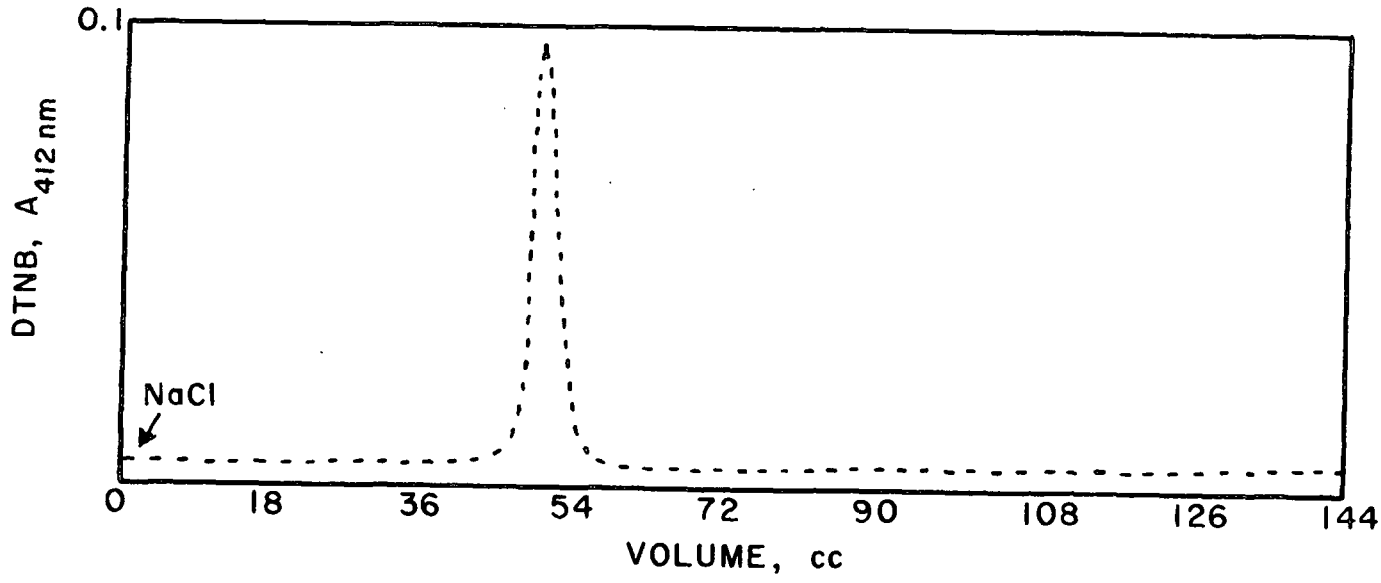



Figure 6. Elution Pattern for Extract of 11(DFN 15)11 on DEAE Sephadex G-25

eluted with dd $H_2O$ and monitored at 254 nm giving the response shown in Fig 7. Fractions 4, 5, and 6 gave strong responses with DTNB while fraction 7 was moderate. Thus fractions 4, 5, 6, and 7 were pooled and freeze-dried giving 52.2 mg of a white powder.

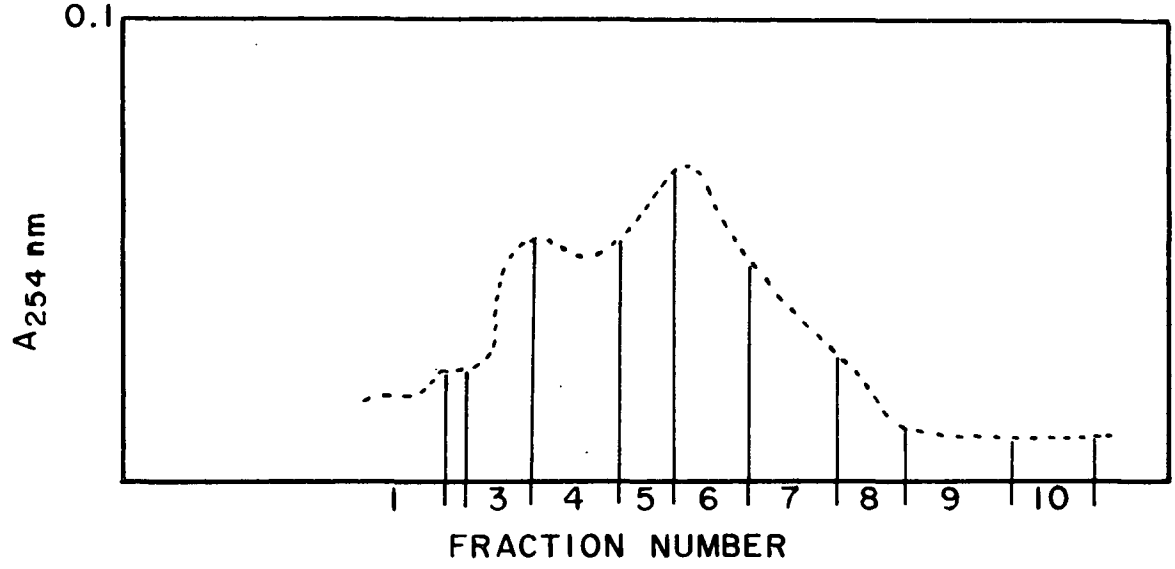



Figure 7. Response of Column Eluate at 254 nm from Douglas-Fir Needle Callus Sample on a Bio-Gel P-2 Column

NINHYDRIN POSITIVE PEAKS FROM AMINO ACID ANALYZER

A portion of this sample was oxidized with performic acid and then subjected to amino acid analysis. This analysis resulted in the four ninhydrin positive peaks shown in Fig. 8.

Two of these peaks can be identified as glycine and ammonia while the other two are unknown. The initial peak has the same retention time as the oxidation products of glutathione ($GSO_3$) and cysteine ($CSO_3$) and is thus the peak of interest. The remainder of the sample was then oxidized with performic acid and applied to the amino acid analyzer. The material corresponding in retention time to this initial peak was collected from the analyzer without being mixed with ninhydrin.

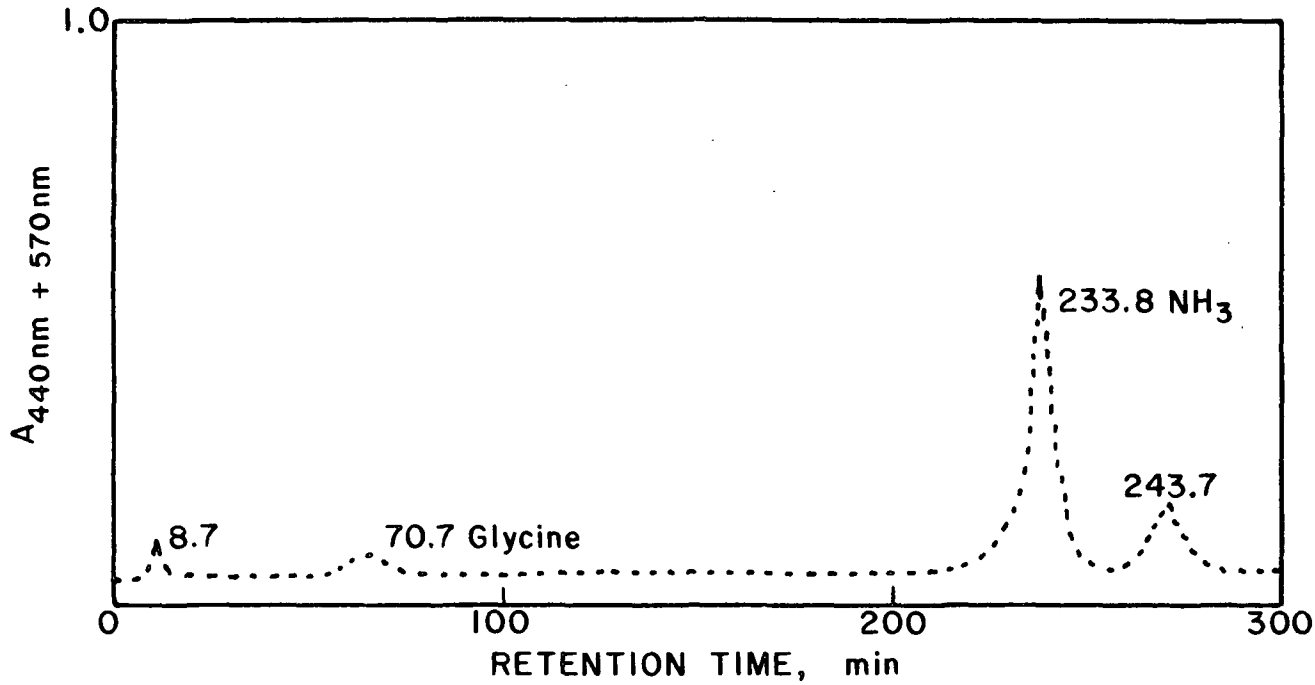



Figure 8. Ninhydrin Positive Peaks from Amino Acid Analyzer

AMINO ACID COMPOSITION OF DOUGLAS-FIR NEEDLE CALLUS SAMPLE COMPARED TO COMMERCIAL GLUTATHIONE

After acid hydrolysis the solution was reapplied to the amino acid analyzer giving the peaks shown in Fig. 9.

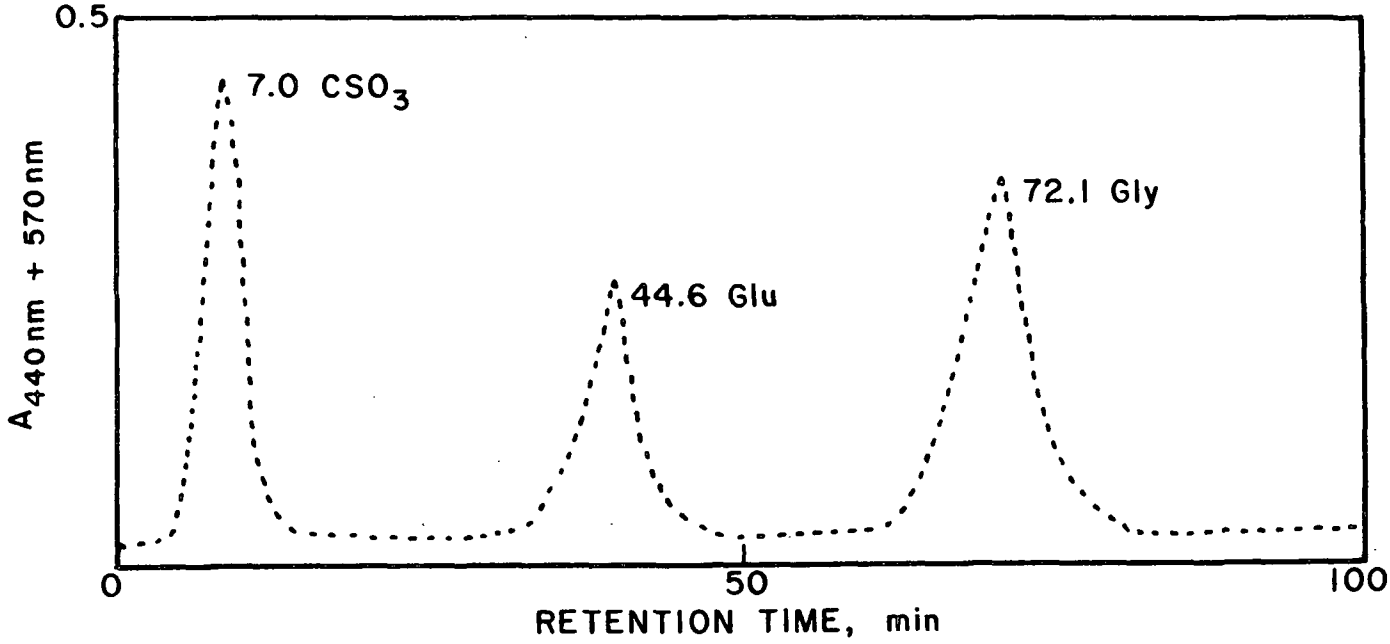



Figure 9. Amino Acid Composition of Major Nonprotein Sulfhydryl Component of Needle Callus

The amino acid composition of commercial glutathione treated in a similiar way is shown in Fig. 10. Integration of the peak areas gives the amino acid ratios shown in Table VIII.

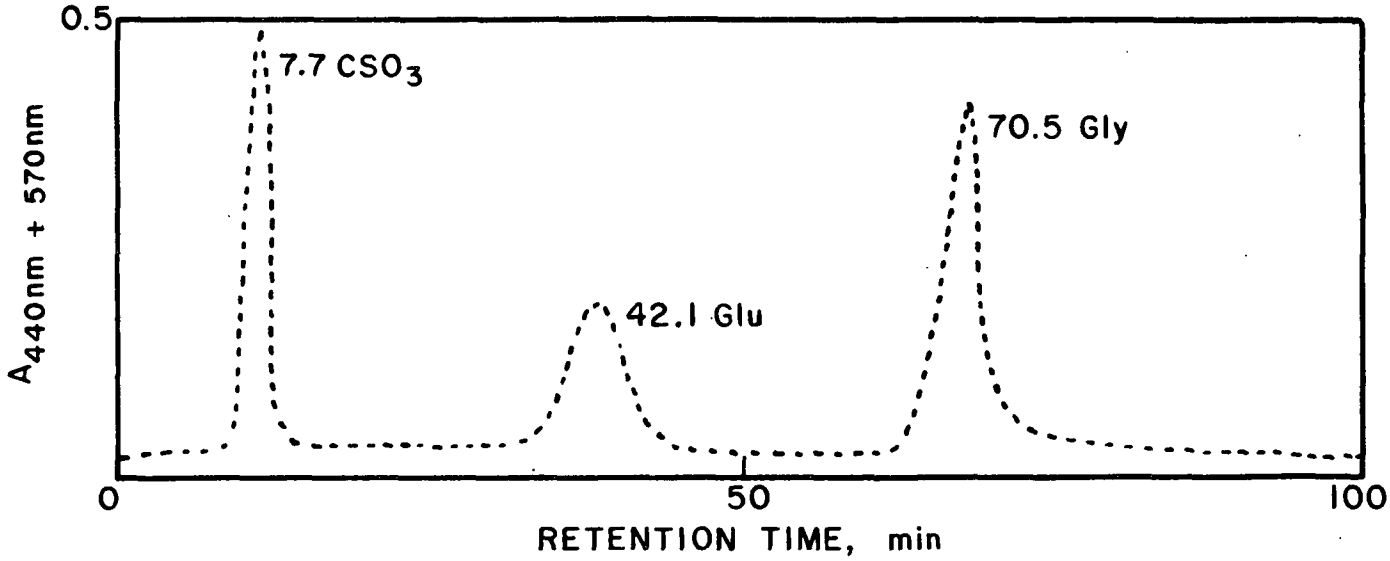



Figure 10. Amino Acid Composition of Acid Hydrolyzed Commercial Glutathione after Performic Acid Oxidation

TABLE VIII

AMINO ACID RATIOS FOR ACID HYDROLYZED DOUGLAS-FIR NEEDLE CALLUS SAMPLE AND COMMERCIAL GLUTATHIONE

| Sample | Cysteine | Glycine | Glutamic Acid |
|---|---|---|---|
| Douglas-fir Needle Callus | 0.96 | 1.00 | 0.58 |
| Commercial Glutathione | 1.08 | 1.00 | 0.39 |
| Glutathione (Hypothetical) | 1.00 | 1.00 | 1.00 |

Acid hydrolysis under these conditions lowers the recovery of glutamic acid from both the sample and commercial glutathione. This effect has been observed by other workers (114).

On the basis of the elution volume from the DEAE Sephadex G-25 column as well as the amino acid composition, the major nonprotein sulfhydryl component of Douglas-fir needle callus is glutathione. This finding allows

glutathione to assume control over the SH/SS redox equilibrium intracellularly in Douglas-fir needle callus. It can do this by exerting its well known "euphoristic" effect on SH containing enzymes (93). Glutathione can protect SH groups on enzymes either by preferentially oxidizing or by reducing SS groups via GSH-disulfide exchange reactions. Other redox related roles that can be ascribed to glutathione would be its association with ascorbic acid in an alternate respiratory chain as well as the control of o-diphenols by preventing their oxidation to toxic o-quinones. Its presence in needle callus also allows it to act as coenzyme in the glyoxalase reactions. After it was known that glutathione was the major nonprotein sulfhydryl component of Douglas-fir needle callus, the question became, how does it vary quantitatively relative to time since subculture? This question is addressed in the following section.

## FLUCTUATION IN GLUTATHIONE LEVELS AND GLUTATHIONE REDUCTASE ACTIVITY IN DOUGLAS-FIR NEEDLE CALLUS RELATIVE TO SUBCULTURE TIME

Once a week for eight consecutive weeks, four different clones of Douglas-fir needle callus were assayed for glutathione and glutathione reductase levels. The objective of this study was to answer the following four questions:

1) How does the glutathione level fluctuate between subcultures, if at all?
2) Is there a correlation between glutathione levels and glutathione reductase activity?
3) Between what values does the glutathione level fall, for the clones studied, in terms of μmoles/g.f.w.?

4) How do these values for needle callus compare to those observed in spruce needles?

The fluctuation in glutathione levels in the various Douglas-fir needle callus clones is shown in Fig. 11 and 12. Clone 31 exhibits a very regular pattern of glutathione fluctuation while the other clones are quite irregular in their variation. The general trend, however, is to have the sulfhydryl level peak from 1-2 weeks after subculture while the levels are lowest at subculture time.

Glutathione levels and the corresponding glutathione reductase activities are plotted in Fig. 13-16. For clones 15 and 94, which have never produced shoots, the glutathione levels vary in an irregular fashion and the glutathione reductase activities do not correlate well. Clone L-2, a shoot producing clone, shows good correlation between glutathione level and glutathione reductase activity as does the nonshoot producing clone 31. The lack of correlation between these two levels in clones 15 and 94 would seem to indicate that additional factors are affecting endogenous glutathione levels. These additional factors are obviously less effective in clones L-2 and 31. The most obvious factors that would cause a decrease in glutathione levels would be a decrease in NADPH, an increase in respiration via ascorbic acid, or an increase in the synthesis of o-diphenols with their simultaneous oxidation to o-quinones. That the effect is probably due to o-quinone formation is seen in tissue which is not subcultured once a month. After five to seven weeks from subculture the tissue turns brown, probably due to o-quinone complexing to protein, and the glutathione levels fall to almost zero. This is followed by the death of the tissue.

Since the glutathione level does not closely follow glutathione reductase activity in clones 15 and 94, the masking effect is probably due to the synthesis

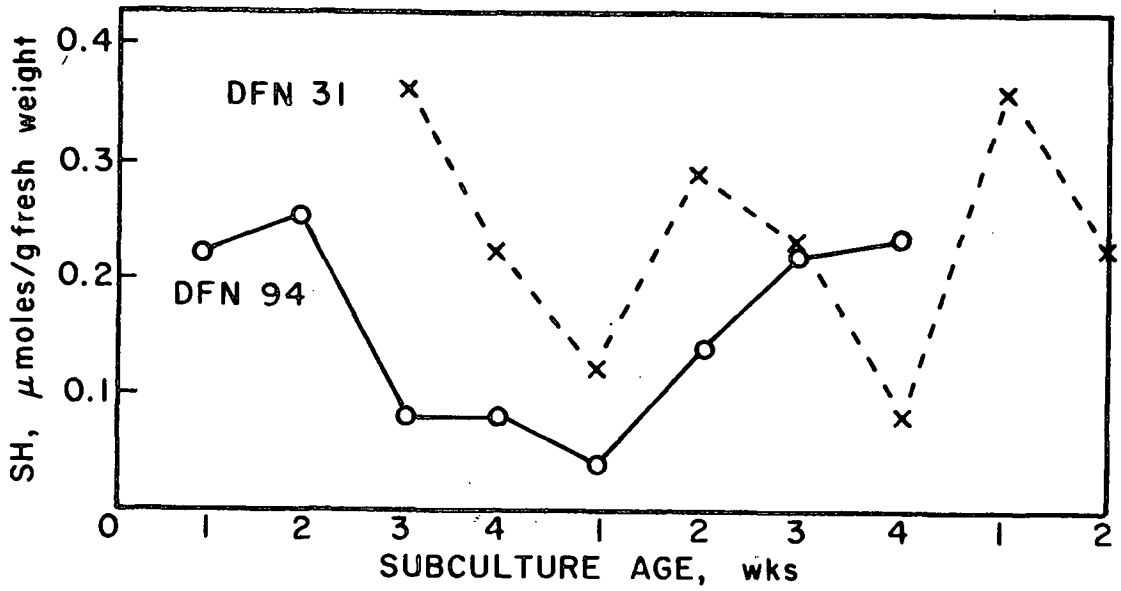



Figure 11. Sulfhydryl Fluctuation in Clones 31 and 94

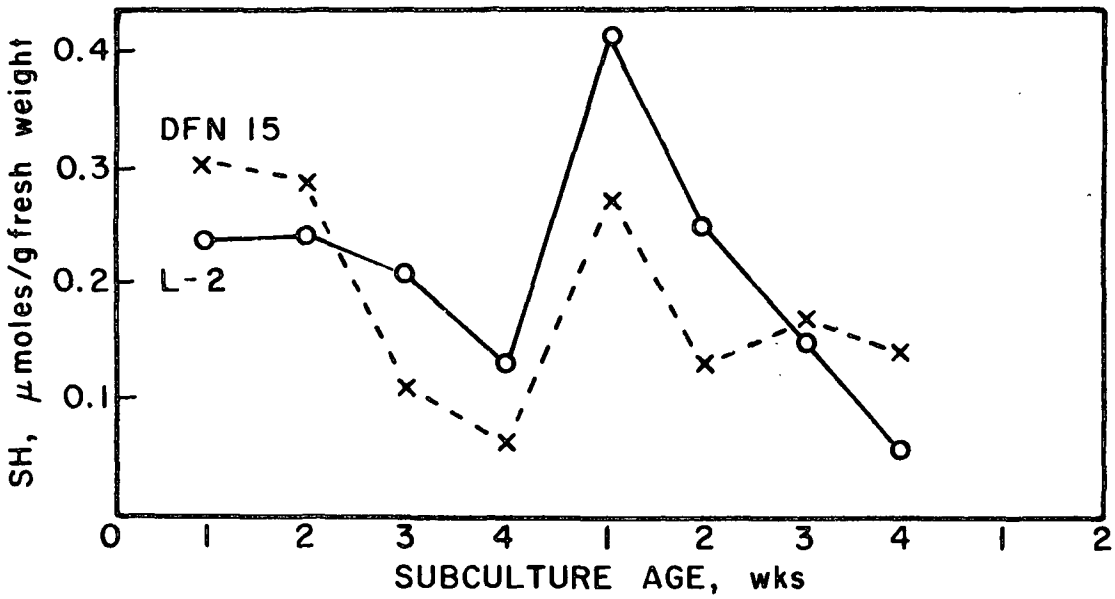



Figure 12. Sulfhydryl Fluctuation in Clones 15 and L-2

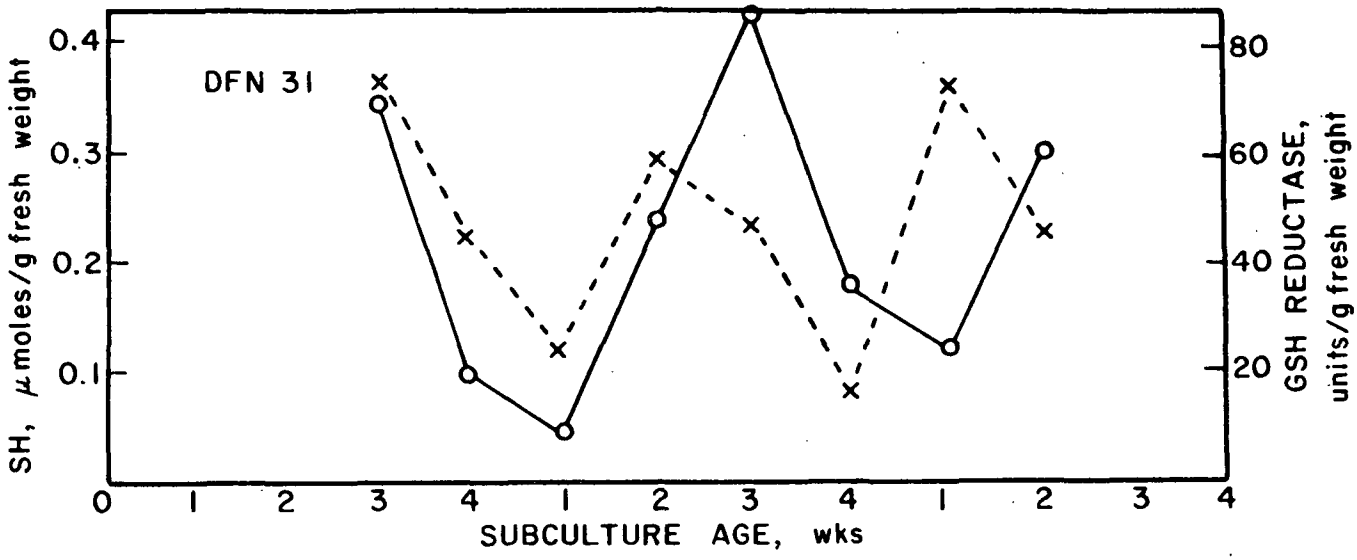



Figure 13. Glutathione — and Glutathione Reductase — Levels in DFN 31

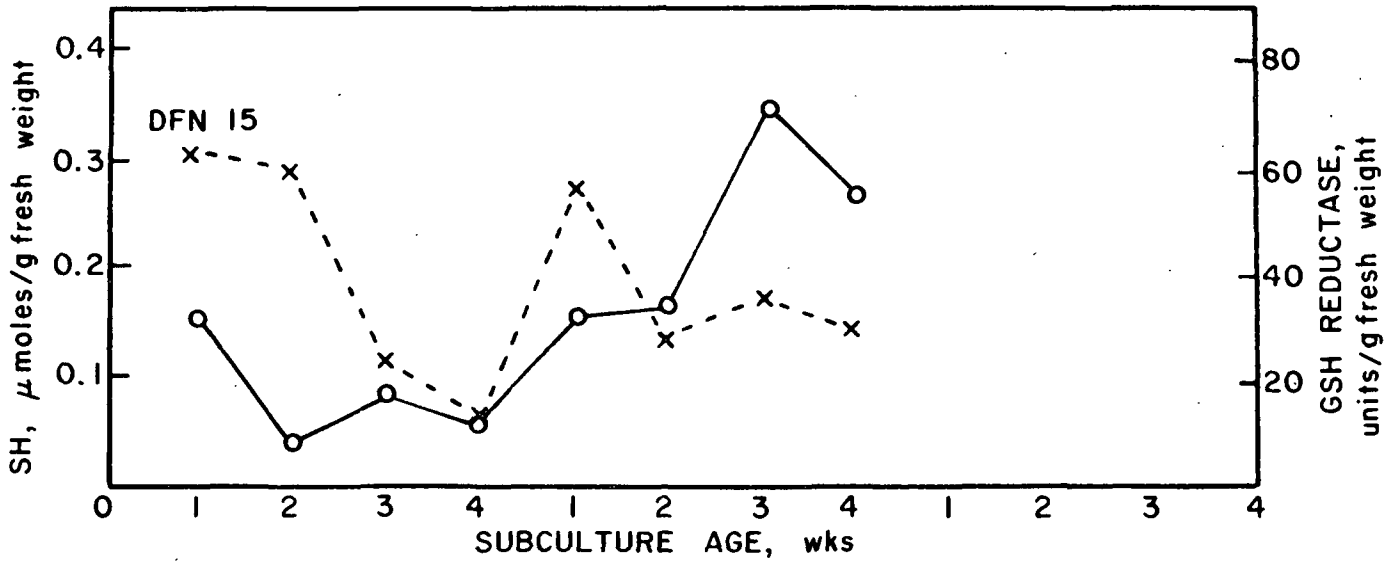



Figure 14. Glutathione — and Glutathione Reductase — Levels in DFN 15

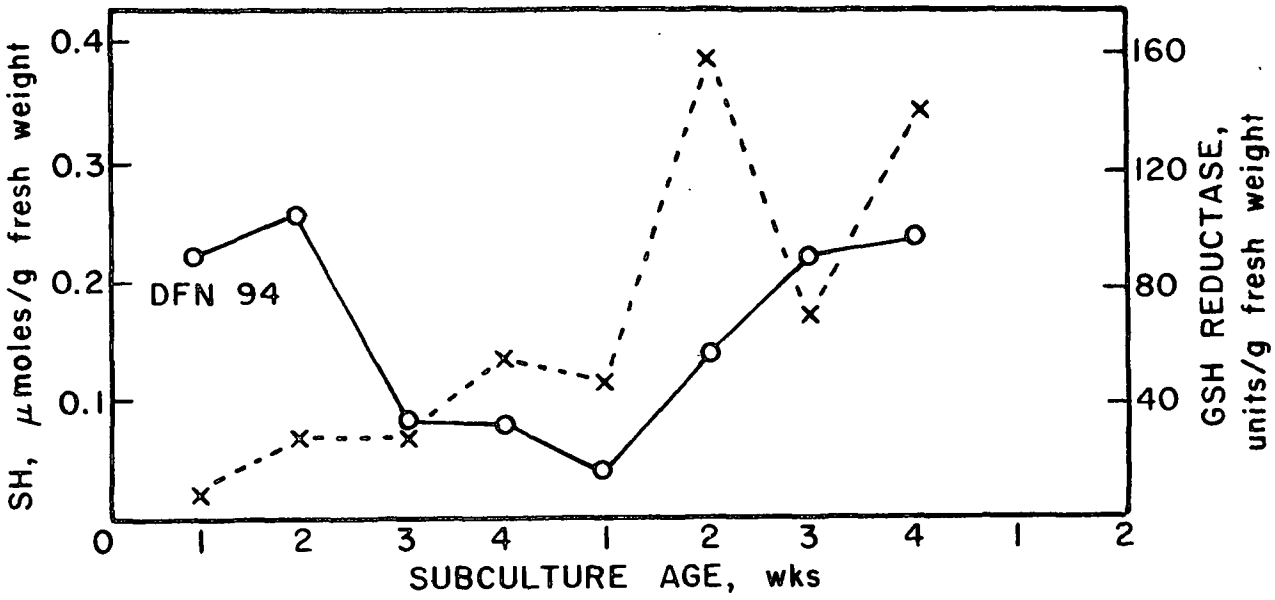



Figure 15. Glutathione — and Glutathione Reductase — Levels in DFN 94

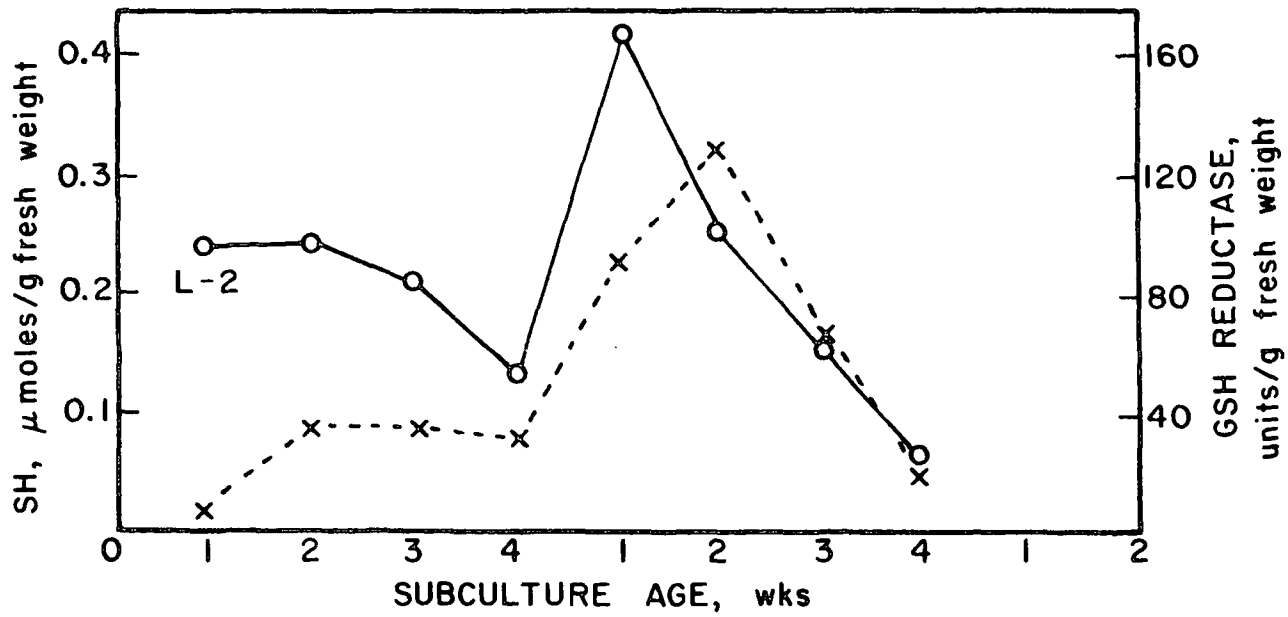



Figure 16. Glutathione — and Glutathione Reductase — Levels in L-2

of o-diphenols. Control of redox potential in these clones cannot occur then in the normal manner. Reducing power in the form of glutathione must be diverted to o-quinones to prevent their build up and eventual browning of the tissue. Clones L-2 and 31, on the other hand, may not have as great a problem with phenolics and their redox potential can be regulated by more normal metabolic events. Since L-2 has produced shoots in the past, clone 31 may also be a good candidate because of its lack of this additional complicating factor.

The values between which the glutathione levels fluctuate in Douglas-fir needle callus (0.08-0.40 μmoles/g.f.w.) are strikingly within the same range in which spruce needles vary (102). This comparison is shown in Fig. 17. The idea that rapid growth and the juvenile state are generally associated with a high SH content of cells (49,99,100) is supported by the finding of high quantities of glutathione in the very young needles in April. The rapidly growing unorganized needle callus cells, however, attain a level no higher than that in the young spruce needles on a fresh weight basis.

The glutathione levels do fluctuate then in Douglas-fir needle callus. While some clones vary in a regular fashion, such as clone 31, most are irregular. The cyclic variations demonstrated, however, indicate that subculturing seems to induce an increase in glutathione levels. Since endogenous IAA levels in this tissue have been shown to be high (115), the action of synthetic auxins such as NAA may be to induce high levels of glutathione. This mode of action for synthetic auxins may be through the alternate respiratory chain for it is known that synthetic auxins inhibit ascorbic acid oxidase activity (116,117). Inhibition of ascorbic acid oxidase could block this respiratory chain with a concomitant buildup of reduced glutathione. The reduced glutathione could exert its general "euphoristic" effect on SH containing enzymes as well as keeping the

o-diphenols reduced. These o-diphenols could have the additional effect, as suggested by Stonier (118,119), of acting as endogenous auxin protectors. This might explain the need of this tissue for exogenous auxins when the endogenous levels appear sufficiently high. The exogenous auxin seems to increase reduced glutathione levels which would prevent o-quinone formation with its resultant browning and eventual death of the tissue. This type of possible interaction is depicted in Scheme X.

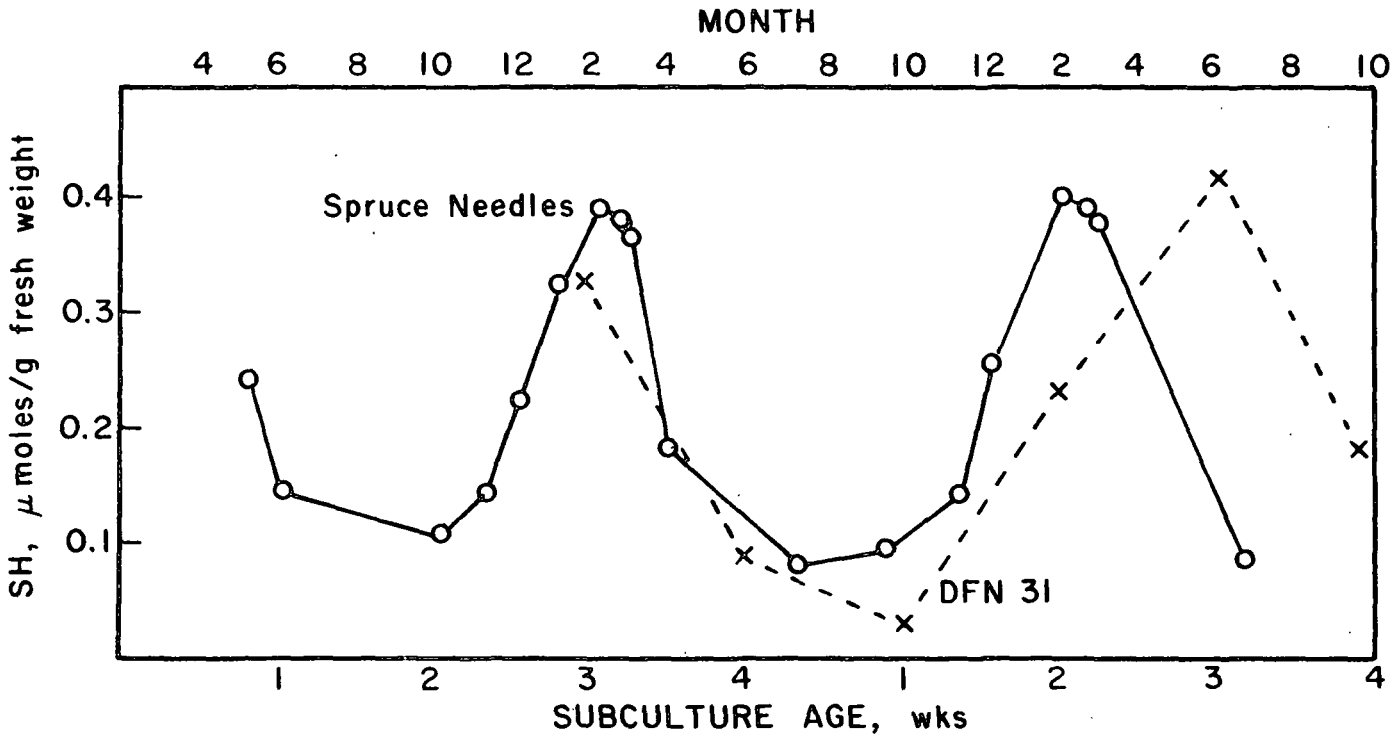
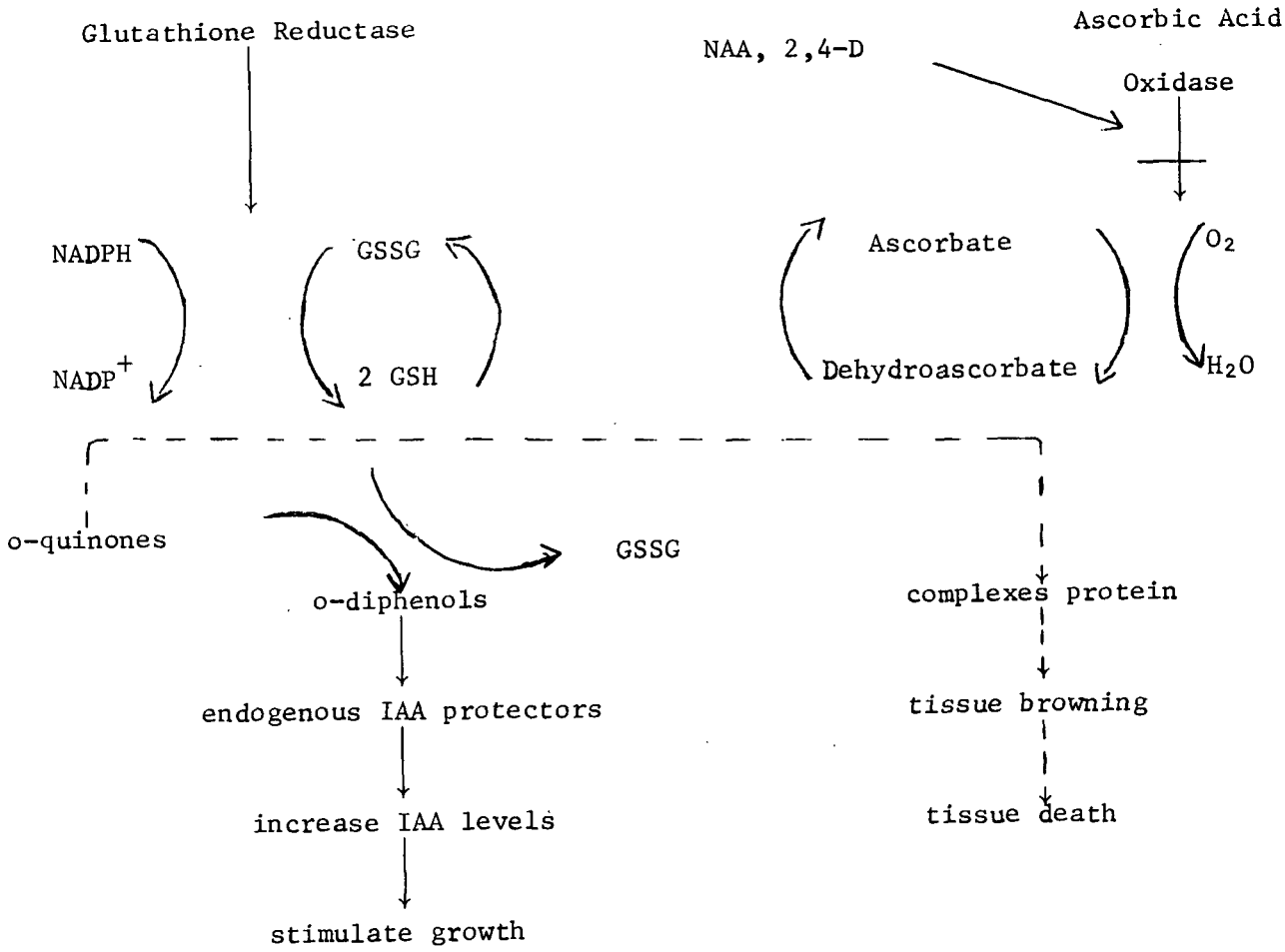



Figure 17. Glutathione Levels in Spruce Needles versus DFN 31

## IDENTIFICATION OF METHYLGLYOXAL IN DOUGLAS-FIR NEEDLES AND ITS ABSENCE IN NEEDLE CALLUS

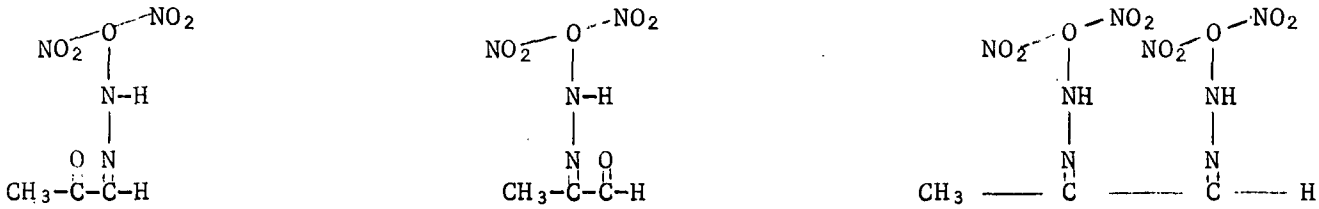
In attempting to isolate methylglyoxal from the two tissue sources, two possible sites could be examined in each. The soluble enzymatic proteins could be extracted and analyzed or the insoluble structural proteins could be analyzed. For a number of reasons a structural protein extract became the sample of choice. For example, a structural protein extract could be prepared

free of a number of interfering substances and there was a recent successful isolation of methylglyoxal from beef liver structural protein by Fodor et al. (76). Thus, insoluble protein extracts were prepared from Douglas-fir needles and Douglas-fir needle callus. Since the needles are viewed as representative of the resting state in this work, their structural protein would be expected to contain methylglyoxal whereas the needle callus would not.




Scheme X. Possible Effect of Synthetic Auxins on Douglas-Fir Needle Callus Metabolism

The reaction of 2,4-DNPH with methylglyoxal can result in three different derivatives based on reaction conditions. The two isomeric monosubstituted hydrazones as well as the di-substituted osazone may be obtained. With excess of 2,4-DNPH the osazone is the predominant derivative. The three possible derivatives are shown in Fig. 18.



Hydrazones Osazone

Figure 18. The 2,4-DNPH Derivatives of Methylglyoxal

After derivatization and extraction with ethyl acetate, the residue from the benzene extract was dissolved in chloroform for both the Douglas-fir needle and needle callus samples. This chloroform fraction, containing a number of derivatized carbonyl compounds, was chromatographed on Silicagel GF plates in toluene:petroleum ether:ethyl acetate (34:5:7) as shown in Fig. 19.

The chromatogram shows an area of considerable size corresponding in $R_f$ to that of the commercial methylglyoxal osazone for the needle sample. No such spot exists for the needle callus sample at the same $R_f$ value. The needle callus sample shows areas corresponding to the reagent itself and the acetone derivative. The osazone area of the needle sample was removed and rechromatographed in two additional solvent systems. The clean single band corresponding in $R_f$ to that of the known methylglyoxal osazone was then removed and characterized.

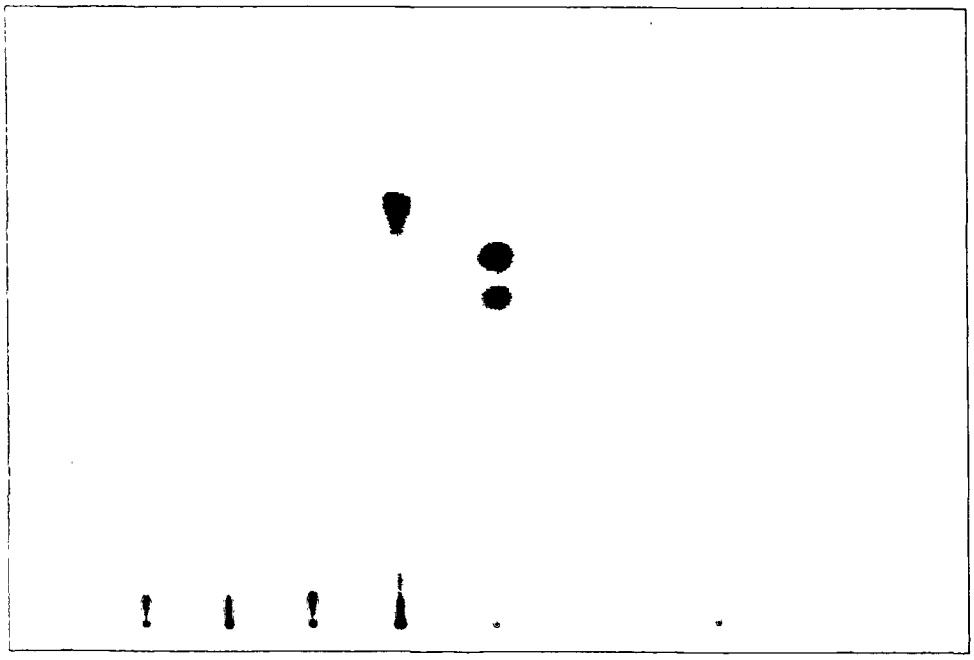


Figure 19. Chromatogram of 2,4-DNPH Derivatives on Silicagel GF Traces from Right to Left; 2,4-DNPH Reagent; Acetone; Commercial Methylglyoxal; Douglas-fir Needles; and Second from Left Douglas-fir Needle Callus

The material was first quantitated by the method of Wells (120). The value obtained was 4.5 X $10^{-9}$ moles of methylglyoxal per gram fresh weight of needles. This was followed by an IR spectrum in Nujol and is compared to that of the known methylglyoxal osazone as is shown in Fig. 20. Although the scales on these spectra do not match exactly, the spectra are nevertheless comparable. Additional characterization of the material was obtained by comparing the spectra to that of the known material in methanolic KOH and chloroform. These spectra are shown in Fig. 21 and 22. Again good spectral matches were obtained.

Finally the melting point was determined and found to be 303° uncorrected as compared to a reported melting point of 309° (52). All of the characterization data for methylglyoxal are summarized in Table IX.

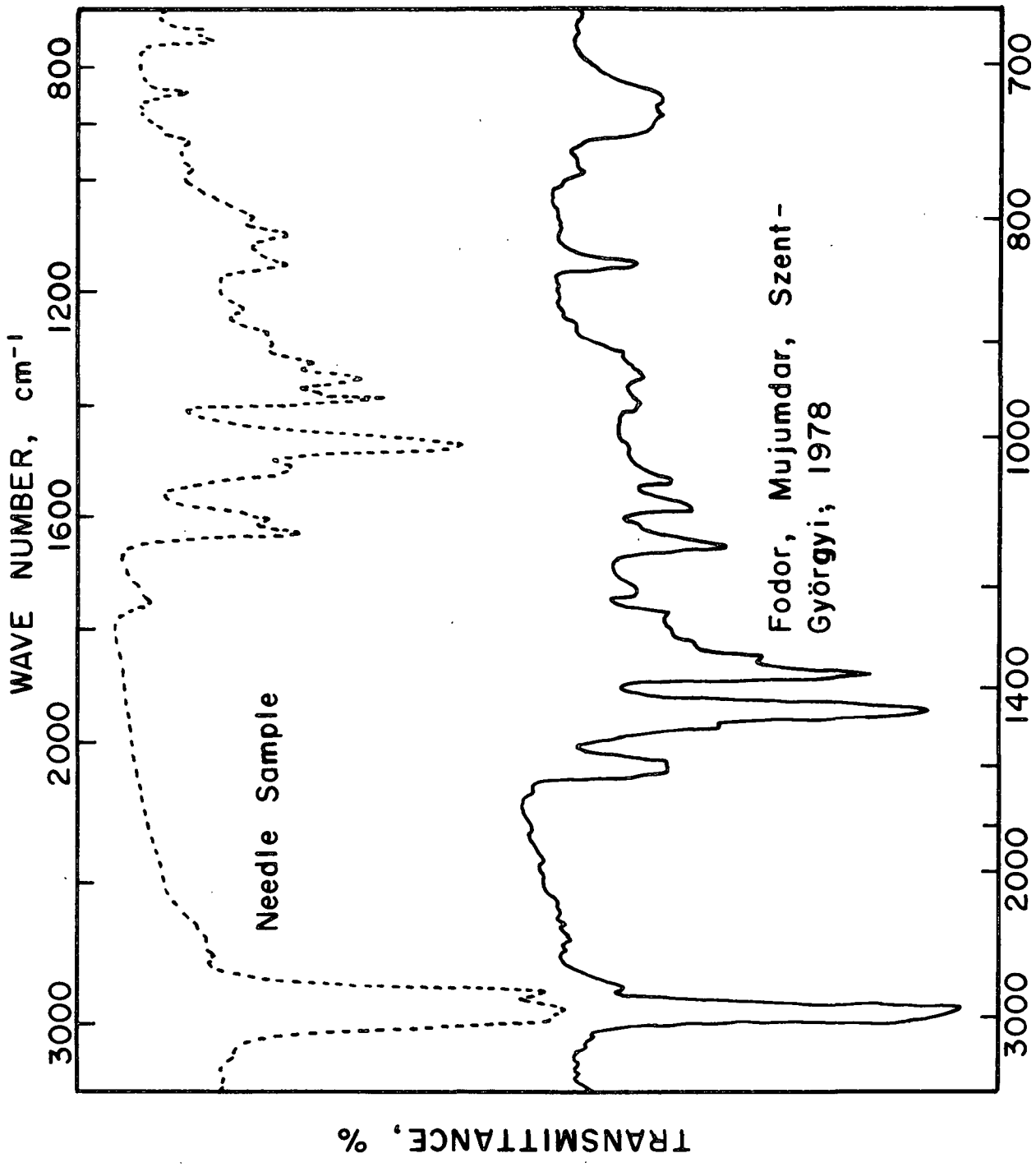



Figure 20. IR Spectra of Methylglyoxal Osazone from Douglas-Fir Needles and Commercial Methylglyoxal by Fodor et al. (76)

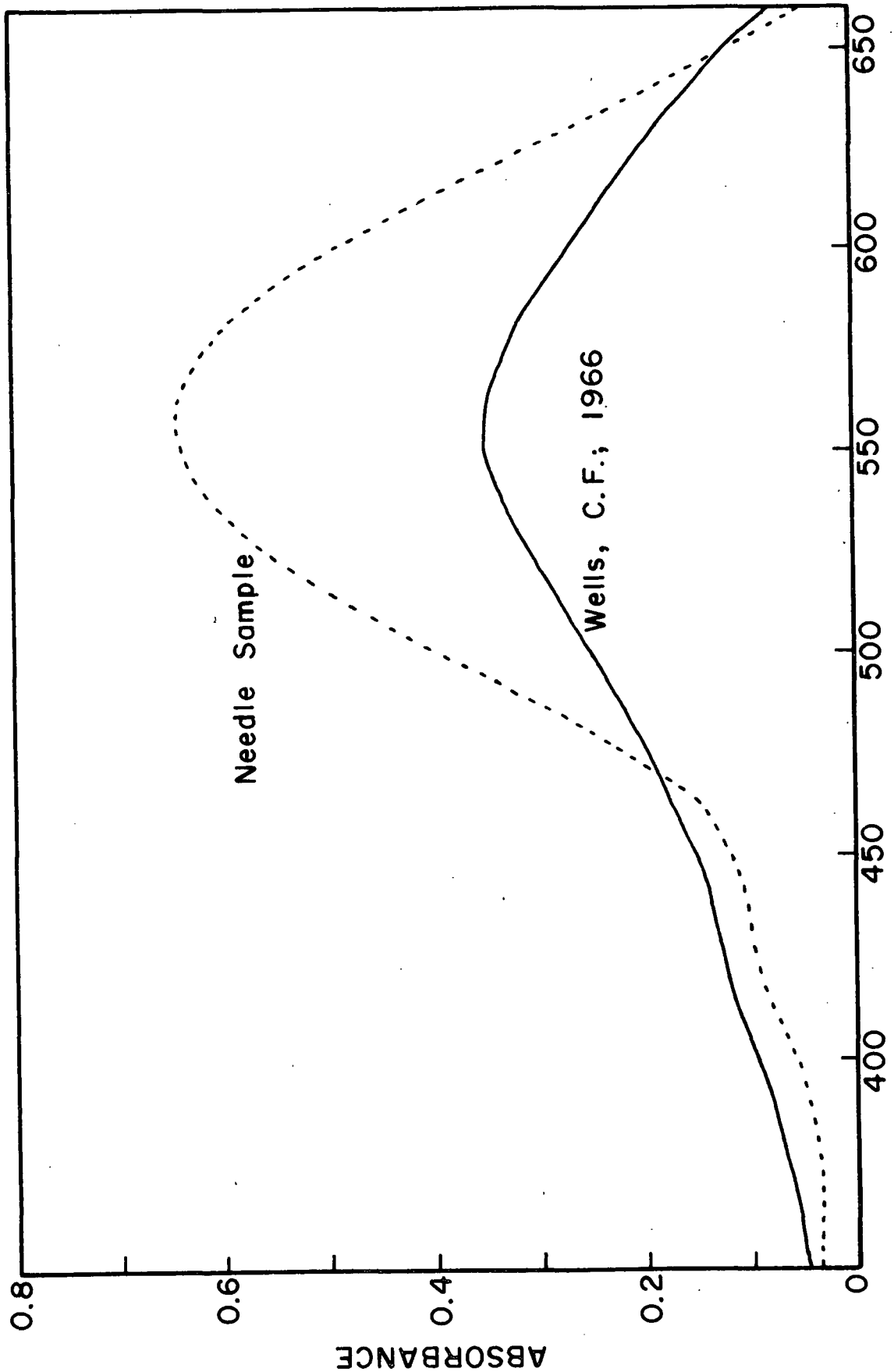



Figure 21. Visible Spectra in Methanolic KOH of Douglas-Fir Needle Sample and Known Methylglyoxal Osazone by Wells (120)

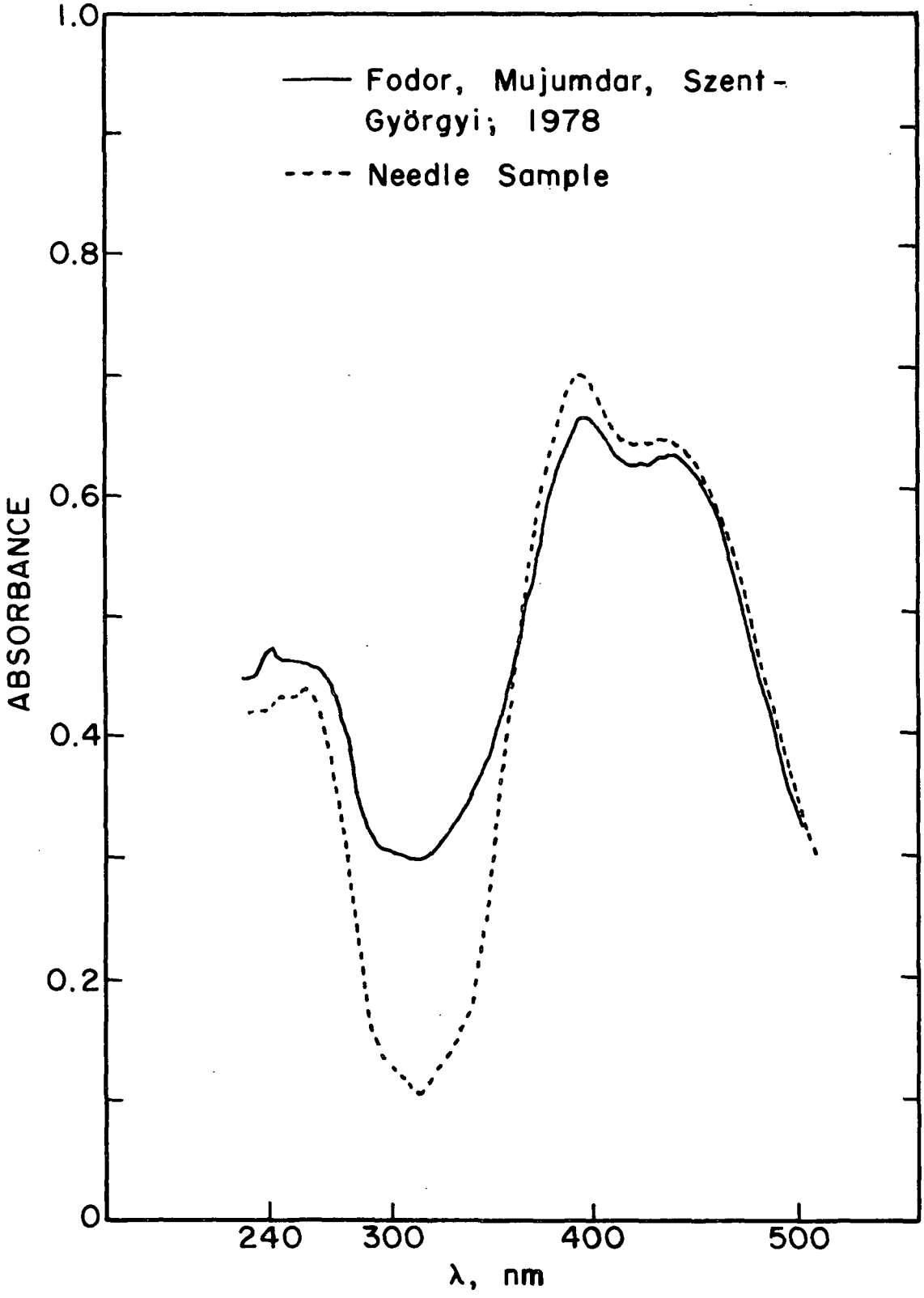



Figure 22. UV-VIS Spectra of Douglas-Fir Needle Sample and Known Methylglyoxal Osazone by Fodor et al. (76) in Chloroform

TABLE IX

SUMMARY OF THE CHARACTERIZATION DATA OF THE DOUGLAS-FIR NEEDLE SAMPLE AND THE KNOWN METHYLGLYOXAL OSAZONE

| Criteria | Literature Value | Known Osazone | Needle Sample |
|---|---|---|---|
| Color | orange-red | orange-red | orange-red |
| Color in Alkali | purple | purple | purple |
| Visible Spectra in MeOH/KOH | $A_{max}$ = 555 | $A_{max}$ = 555 | $A_{max}$ = 555 |
| | $A_{shld}$ = 425 | $A_{shld}$ = 425 | $A_{shld}$ = 425 |
| UV-Visible Spectra in Chloroform | $A_{max}$ = 265,390,440 | $A_{max}$ = 265,390,440 | $A_{max}$ = 265,390,440 |
| | $A_{min}$ = 315 | $A_{min}$ = 315 | $A_{min}$ = 315 |
| $R_f$ Silicagel GF | | | |
| toluene:pet:ether: ethyl acetate (34:5:7) | - | 0.675 | 0.675 |
| $R_f$ Silicagel GF | | | |
| benzene:chloroform (2:1) | - | 0.160 | 0.160 |
| Melting Point | 309° | - | 303° uncorrected |

The Douglas-fir needle callus sample consisted of a mixture of 224.20 g.f.w. of DFN 161-2 and 183.10 g.f.w. of DFN 53. No methylglyoxal derivative could be identified from this sample.

To examine the possible interference of certain carbohydrate and lignin moieties in the isolation procedure, a series of additional substances was tested. Thus, solutions of dioxane lignin, cellulose, xylan, and arabinogalactan were treated with 2,4-DNPH in the same manner as the needle and needle callus samples. Spectral studies of these samples showed no interference. It is thus very likely that the methylglyoxal found in the needles was complexed to the structural proteins via a charge-transfer reaction.

The presence of methylglyoxal in an insoluble fraction from Douglas-fir needles and its absence from a similar Douglas-fir needle callus preparation lends considerable support to Szent-Gyorgyi's idea that it plays a significant role in cellular regulation. Its presence in needles could allow it to assume an inhibitory or "brakelike" action on cell division whereas its absence in needle callus could allow the observed proliferation. When considered in light of the isolation of methylglyoxal from live structural proteins (76), these findings indicate that methylglyoxal could also be operating in conifers as a cell division inhibitor.

## EFFECT OF METHYLGLYOXAL AND GLUTATHIONE ON THE GROWTH OF DOUGLAS-FIR SUSPENSION CELLS

The previous section demonstrated the existence of methylglyoxal in Douglas-fir needles and its absence from needle callus. If methylglyoxal does indeed play a role in cellular regulation as a division inhibitor, then addition to needle callus might be expected to inhibit growth. To study this possible effect calli from 71 plates of 11(DFN 104) were placed in liquid media and the growth curves determined at various levels of methylglyoxal.

Because of the relationship between redox potential and cell division, a portion of these suspension cells were also subjected to glutathione treatments at various levels. By increasing the reducing environment in this way a number of important factors could be studied. For example, does added reducing power in the medium induce greater growth or reduce the lag phase of the growth curve? On the other hand might glutathione inhibit the growth of the cells as has been reported by Bergmann and Rennenberg (121) in soybean callus cultures. These workers found a 50% inhibition of growth with reduced glutathione at $10^{-5}$M. The effects of methylglyoxal and glutathione growth curves are shown in Fig. 23 and 24.

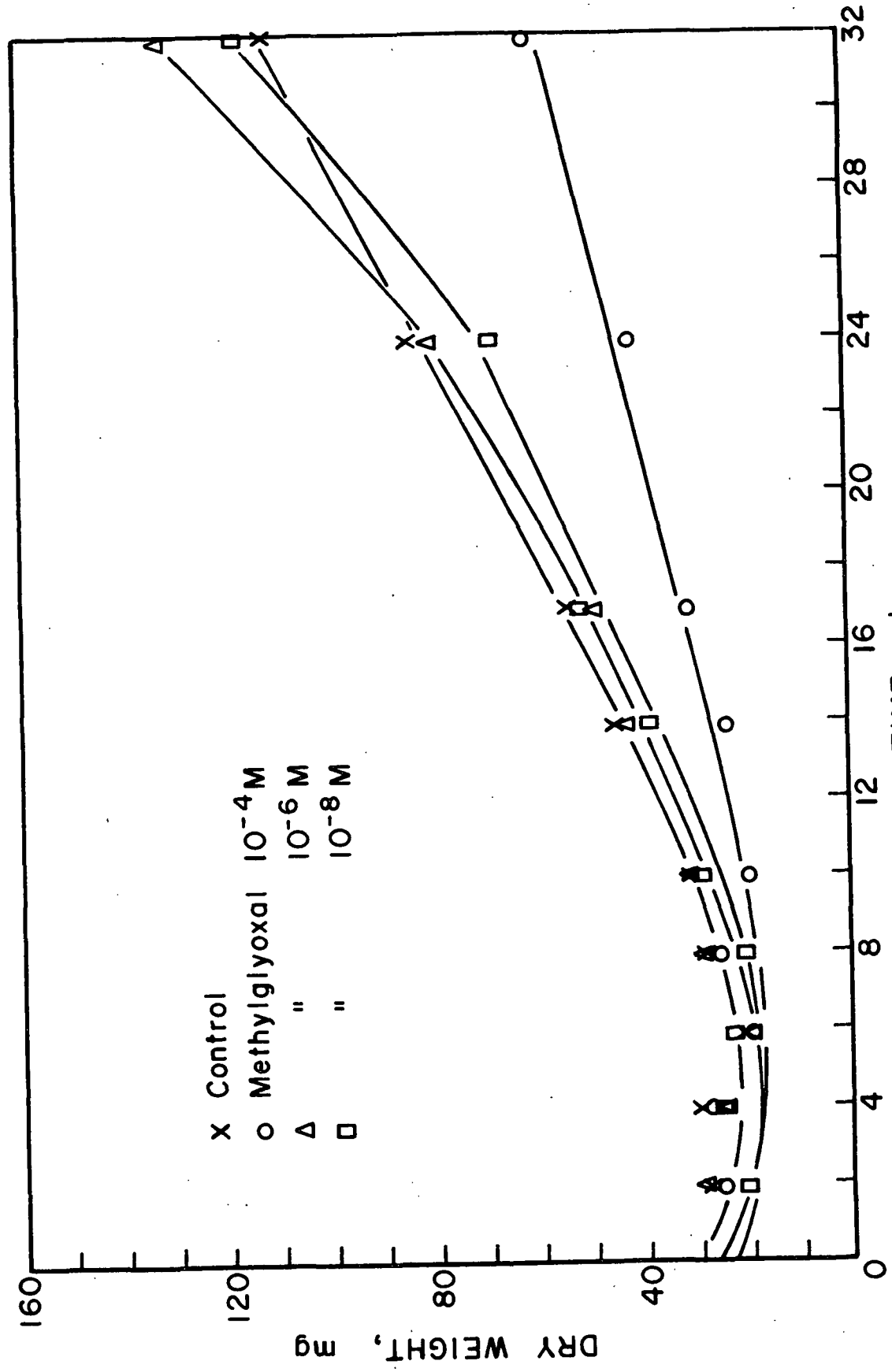



Figure 23. Effect of Methylglyoxal on the Growth of 11(DFN 104) Suspension Cells

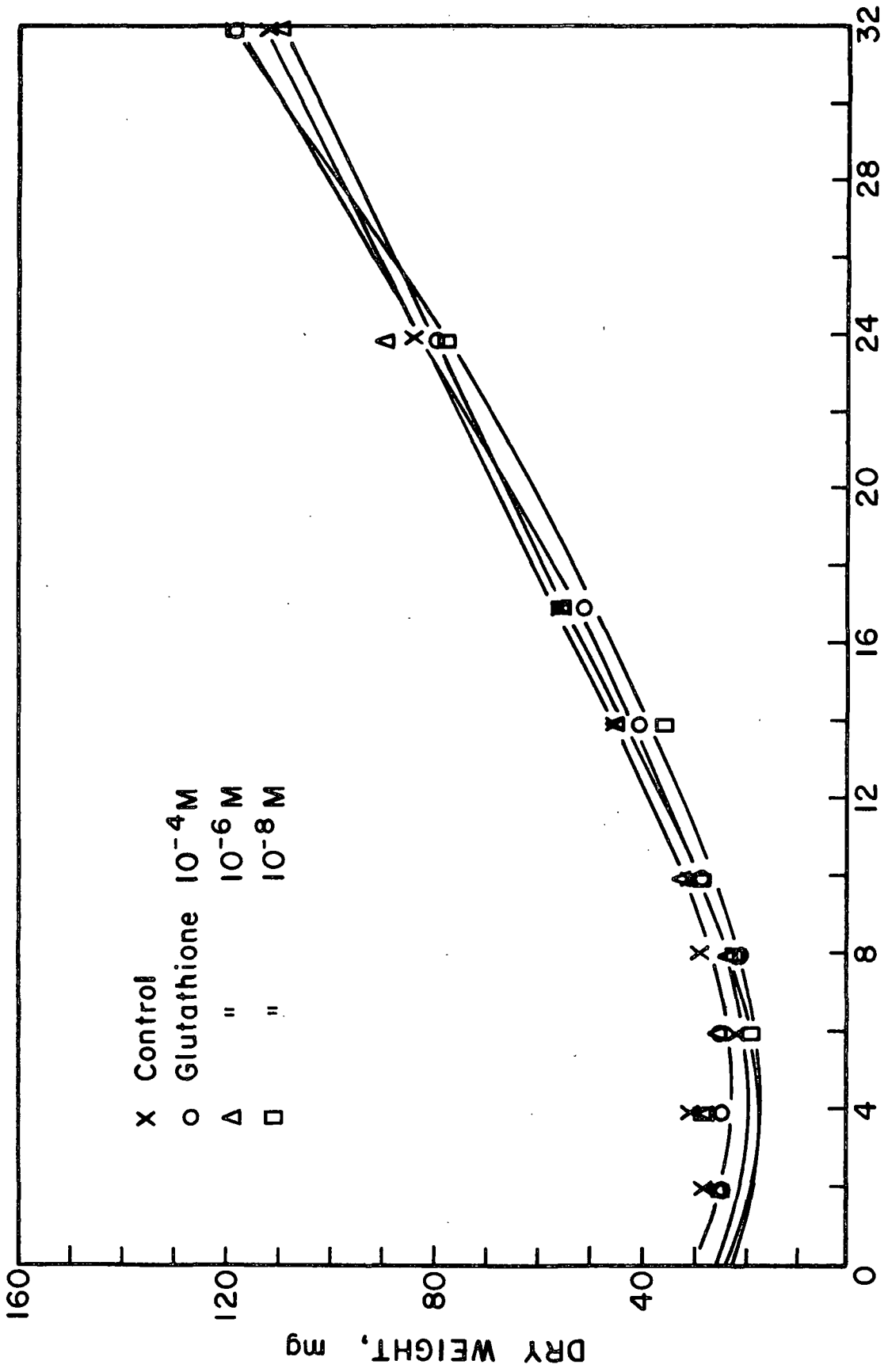



Figure 24. Effect of Glutathione on the Growth of 11(DFN 104) Suspension Cells

The graphs on the preceding pages show the effect of methlyglyoxal and glutathione on the growth of Douglas-fir needle callus suspension cells. The growth, as represented by dry weight increase, covers a 32 day period beginning with the time of removal from agar. Since dry weights were measured nothing about cell division can be inferred. To check the treatments for any statistically significant differences from the control, the dry weight means of the final sampling data were subjected to Duncan's Multiple Test. The results showed that methlyglyoxal at $10^{-4}$M was indeed inhibitory, while the other concentrations were not significantly different from the control. Reduced glutathione meanwhile showed no statistically significant difference at any concentration.

Methylglyoxal, then, does have an inhibitory effect on the growth of needle callus suspension cells. At the $10^{-4}$M level, the inhibition is approximately 50%. Glutathione however does not show any effect on growth as measured by dry weight increase at $10^{-4}$, $10^{-6}$, or $10^{-8}$M. These results indicate that, in the modified MS medium used, the intracellular redox potential appears to be at an optimum for growth. It must be stressed however that reduced glutathione is not very stable, having a half-life of 4 days in a nipple flask containing just medium and about 4 hours in the same system with cells.

## METHYLGLYOXAL METABOLIC PATHWAYS IN DOUGLAS-FIR NEEDLES, NEEDLE CALLUS, AND TOBACCO TISSUES

In a previous section the presence of methylglyoxal in Douglas-fir needles and its absence in needle callus has been reported. It would seem to follow from this finding that methylglyoxal synthetase, the enzyme converting dihydroxyacetone phosphate to methylglyoxal, should also be present in needles but not in needle callus. An early investigation into this possibility showed it to be true. The callus clone 16(DFN 94)11 did not contain methylglyoxal

synthestase activity, whereas needles did contain enzyme activity. The needles gave a value of 1.17 units/g.f.w. While this value is quite low, it is important to note that the analysis was done in mid-April. At this time, the needles analyzed were a collection of mature needles as well as varying stages of developing needles. The importance of this observation will be clear in a following section.

Three methylglyoxal enzymatic catabolic pathways are known to exist. One of these is the direct oxidation of methylglyoxal to pyruvate by α-ketoaldehyde dehydrogenase. This enzyme was not found in needles or needle callus nor was D,L-lactaldehyde dehydrogenase, therefore, these enzymes will not be discussed any further. The other two enzymes, glyoxalase and methylglyoxal reductase, were both found in the needle callus tissue as well as in tobacco callus and crown gall tissues. Crude needle enzyme preparations exhibited methylglyoxal reductase activity but no glyoxalase I or II. Isoelectric focusing, however, freed glyoxalase I from its inhibitor.

Glyoxalase activity in needle callus did fluctuate between subcultures. Depending on the time since subculture as well as the clone, the glyoxalase I activity ranged from 550-1400 units/g.f.w. and glyoxalase II from 100-650 units/g.f.w. The ratio of glyoxalase I to glyoxalase II remained very constant during the subculture period as is shown in Fig. 25. Glyoxalase activity is summarized in Table X.

The data to this point may be summarized as follows: an insoluble fraction from Douglas-fir needles was shown to contain methylglyoxal, while the same fraction from Douglas-fir needle callus did not. Additional evidence for the validity of this finding was provided when methylglyoxal synthetase activity was discovered in needles but not in needle callus.

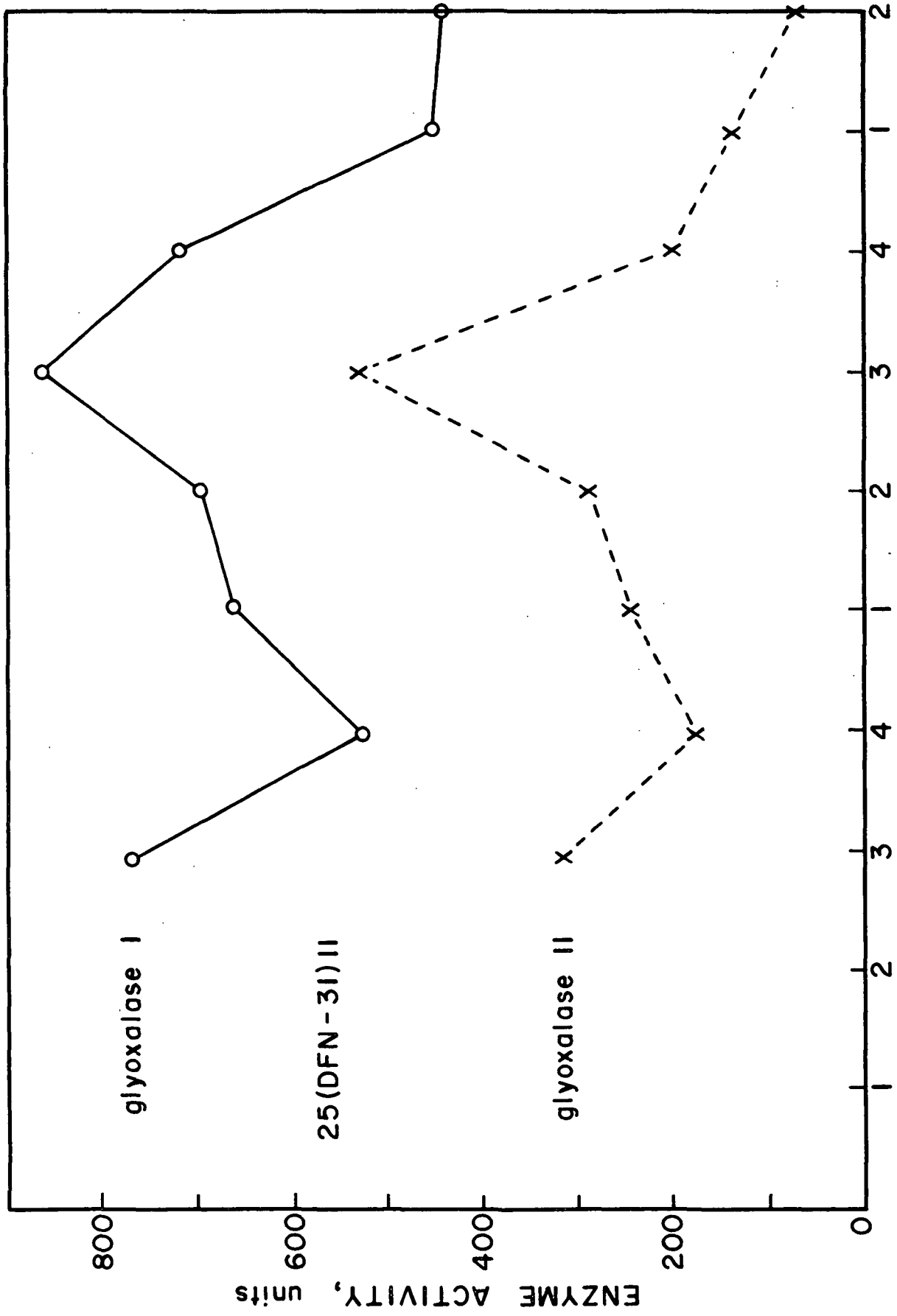



Figure 25. Glyoxalase I and II Fluctuations in 25(DFN 31)11

TABLE X

GLYOXALASE I AND II ACTIVITY IN VARIOUS DOUGLAS-FIR NEEDLE CALLUS CLONES RELATIVE TO SUBCULTURE TIME. THE RESULTS ARE GIVEN IN UNITS PER GRAM FRESH WEIGHT AS THE MEANS OF THREE DETERMINATIONS

| Sample | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 |
|---|---|---|---|---|---|---|---|---|---|---|
| 6(L-2)11 | | | | | | | | | | |
| Glyoxalase I | 807 | 825 | 1323 | 895 | 1146 | 1286 | 1399 | 1038 | | |
| Glyoxalase II | 212 | 245 | 354 | 315 | 391 | 425 | 478 | 341 | | |
| 26(DFN 94)11 | | | | | | | | | | |
| Glyoxalase I | 1079 | 666 | 940 | 511 | 688 | 1055 | 1194 | 1250 | | |
| Glyoxalase II | 324 | 296 | 486 | 400 | 364 | 657 | 644 | 652 | | |
| 10(DFN 15)11 | | | | | | | | | | |
| Glyoxalase I | 848 | 433 | 600 | 555 | 537 | 717 | 1045 | 1366 | | |
| Glyoxalase II | 244 | 138 | 231 | 94 | 268 | 326 | 498 | 334 | | |
| 25(DFN 31)11 | | | | | | | | | | |
| Glyoxalase I | | | 769 | 519 | 668 | 695 | 866 | 723 | 455 | 441 |
| Glyoxalase II | | | 308 | 173 | 231 | 291 | 520 | 189 | 134 | 77 |

The situation relative to methylglyoxal catabolic enzymes is not quite as clear since two routes are possible in the needle callus. The needles contain active methylglyoxal reductase and inhibited glyoxalase I. According to the theory the proliferative needle callus tissue should contain glyoxalase free within the cells whereas the needles should contain bound glyoxalase. The needle callus cells do contain glyoxalase and are in accordance with the theory. The existence of methylglyoxal reductase in the needle callus allows a secondary route for releasing the methylglyoxal braking action. Depending upon the levels of glutathione or NADPH, the needle callus then has two means of destroying methylglyoxal, even if it were synthesized.

The picture in the needles, however, is a bit more complicated. Being representatives of the "resting state", the needles according to the theory should contain bound glyoxalase. Glyoxalase I activity could not be demonstrated in crude preparations of Douglas-fir needles. The activity was "bound," however, in the sense that it was inhibited in crude preparations. Removal of the inhibition by isoelectric focusing allowed glyoxalase I to be expressed in needles. These findings suggest the following situation may be occurring in needles. First, rather than glyoxalase being bound intracellularly, its activity appears to be controlled by an inhibitor. Second, methylglyoxal reductase might play the role of protecting the cell division in normally developing needles from methylglyoxal. The experimental results in the next section help answer this question. However, it must be remembered that these experiments are an <u>in vitro</u> attempt at extrapolating the <u>in vivo</u> situation.

## RELATIONSHIP BETWEN METHYLGLYOXAL REDUCTASE AND SYNTHETASE IN THE DEVELOPING DOUGLAS-FIR NEEDLE

The inability to isolate glyoxalase activity from a crude preparation of Douglas-fir needles led to a study of needle development. Since mature

"nondividing" needles contained no active glyoxalase, the idea was that young developing needles actively undergoing cell division might contain glyoxalase. To test this idea needles were collected from seedlings and divided into three categories. One group consisted of early flush material 1.0-1.5 cm in length, another group was made up of fully elongated flush material 2.5-3.0 cm long. The third group consisted of mature needles 2.5-3.0 cm long.

Hypothetically, the glyoxalase activity might be expected to decrease linearly with age to a value of zero for the mature needles. Accompanying this change, the methylglyoxal synthetase activity should peak in the fully elongated flush material to act as a cell division brake. When these three stages of needle development were studied, a startling discovery was made. Again no glyoxalase activity could be elicited from any sample. The methylglyoxal reductase activity, however, was present and decreased almost exactly in a linear fashion with the age of the needles. This was precisely the relationship that was expected of the glyoxalase activity. On the other hand, methylglyoxal synthetase activity was highest in the 2.5-3.0 cm fully elongated flush material with no activity in the other needle samples. These results are shown in Fig. 26. A summary of enzyme activities in the various tissue types is given in Table X.

Methylglyoxal seems to play a very definite role in the development of Douglas-fir needles. In the young needles, cell division is protected from the inhibitory braking action of methylglyoxal by methylglyoxal reductase. As the needle develops and reaches its fully elongated stage the methylglyoxal reductase activity decreases and methylglyoxal synthetase activity peaks. This allows the buildup of methylglyoxal which acts as a brake on further cell division. As the needle matures, both of these enzymes are lost and the cells

remain in a resting state. Douglas-fir needle development involves the proper modulation of methylglyoxal reductase and synthetase activities. The glyoxalase activity remains inhibited during all stages of needle development. This data supports the contention of Szent-Gyorgi that methylglyoxal acts as a cell division brake. It differs from the theory, however, by replacing the glyoxalase system with methylglyoxal reductase. Methylglyoxal reductase appears to protect developing needles from the inhibitor methylglyoxal until elongation has been reached.

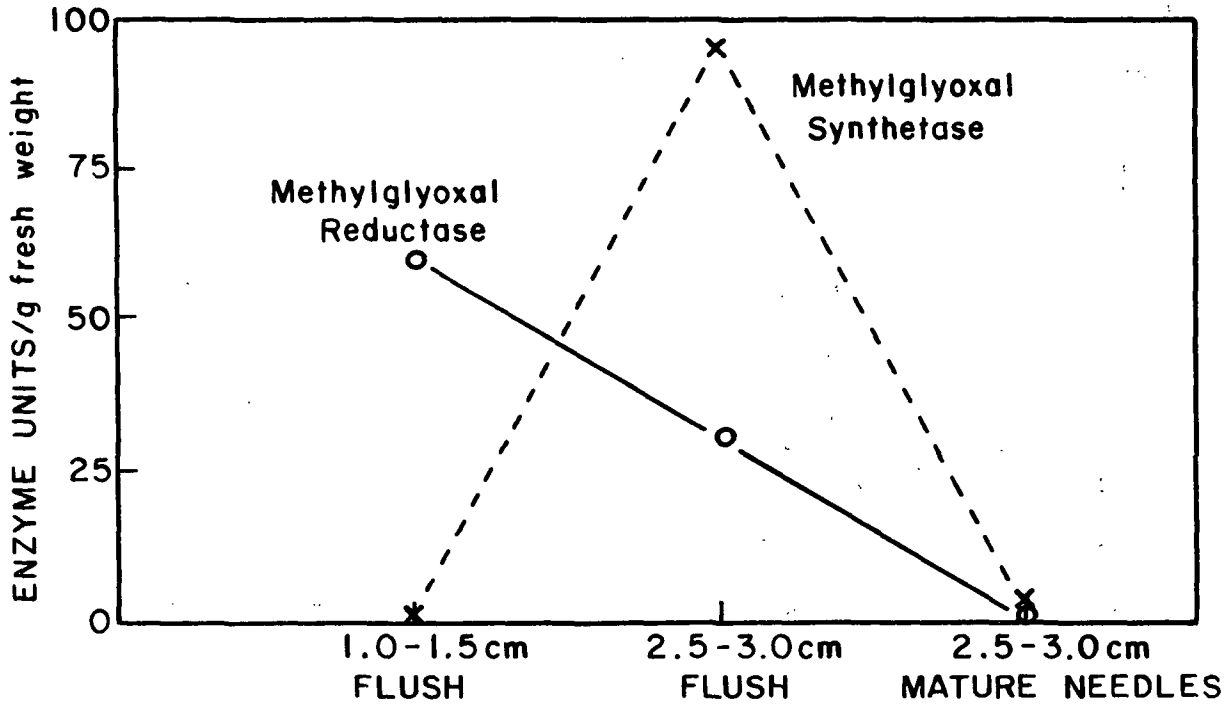



Figure 26. Relationship Between Methylglyoxal Reductase and Synthetase at Various Stages of Douglas-Fir Needle Development

The proliferative Douglas-fir needle callus cells contain no methylglyoxal nor the enzyme to synthesize it. Their cell division is completely unchecked by the brakelike action of methylglyoxal. Furthermore, any methylglyoxal entering the needle callus cells would be rapidly metabolized because of both methylglyoxal reductase and glyoxalase. Since needle callus tissue is the

result of a wounding action, the presence of active glyoxalase in needle callus is most easily viewed as a wound response. Put very simply, the needles contain the genes for the synthesis of glyoxalase. These genes are expressed or translated into protein under conditions of normal development. The glyoxalase I activity, however, is inhibited during these stages. In the event of a wound or callus formation, the inhibitor is rendered inactive allowing glyoxalase activity to be expressed. Subculturing of this material continues to perpetuate the lack of inhibition of glyoxalase activity. With its lack of methylglyoxal and its strong complement of methylglyoxal-degrading enzymes, the tissue is strongly proliferative by Szent-Gyorgyi's criteria.

## METHYLGLYOXAL METABOLIC PATHWAYS IN VARIOUS TOBACCO TISSUES

The tobacco callus and crown gall tissues were very similar in their glyoxalase and methylglyoxal reductase activities to those of Douglas-fir needle callus as shown in Table XI. This lack of methylglyoxal synthetase activity, combined with the already mentioned methylglyoxal catabolic enzymes, makes this tissue likewise representative of the proliferative state as defined by Szent-Gyorgyi. The tobacco stem tissue exhibits methylglyoxal reductase activity at roughly the same level as the young needles. This tissue differs from needle tissue however by giving both glyoxalase I and II activity. On this basis it would seem that normal development of tobacco plants might involve control of both glyoxalase and methylglyoxal reductase activities. Douglas-fir needles, containing inhibited glyoxalase I require further control over methylglyoxal reductase.

TABLE XI

SUMMARY OF ENZYME ACTIVITIES IN CRUDE PREPARATIONS OF DOUGLES-FIR AND TOBACCO TISSUES IN UNITS PER GRAM FRESH WEIGHT AS MEANS OF THREE DETERMINATIONS

| Sample | Glyoxalase I | Glyoxalase II | Methyl-glyoxal Reductase | Methyl-glyoxal Synthetase |
|---|---|---|---|---|
| 1.0-1.5 cm Needles | 0.00 | 0.00 | 57.50 | 0.00 |
| 2.5-3.0 cm Needles | 0.00 | 0.00 | 29.20 | 96.90 |
| 2.5-3.0 cm Old Needles | 0.00 | 0.00 | 0.00 | 0.00 |
| 13(DFN 15)11 | 1250.13 | 652.22 | 199.94 | 0.00 |
| 29(DFN 94)11 | 1044.80 | 498.34 | 225.74 | 0.00 |
| Tobacco Callus | 717.14 | 139.78 | 161.58 | 0.00 |
| Tobacco Crown Gall | 649.30 | 113.60 | 86.20 | 0.00 |
| Tobacco Stems | 452.51 | 58.70 | 59.64 | 0.00 |

GLYOXALASE I ACTIVITY AFTER ISOELECTRIC FOCUSING OF A DOUGLAS-FIR NEEDLE PREPARATION

Since no needle preparation had exhibited any glyoxalase I or II activity, a number of add back experiments were attempted. The purpose of these experiments was to observe the possible inhibitory action of a needle extract on callus and commercial glyoxalase I activity. Such inhibition, if found, would explain the lack of glyoxalase activity from crude needle preparations.

An acetone powder was prepared in the usual manner from 2.9721 grams of 50-week-old needles. The powder was extracted in 5.0 cc of 50 mM potassium phosphate buffer at pH 6.8. This extract strongly inhibited the glyoxalase I

activity from needle callus as well as commercial enzyme. As little as 50 µL of this extract totally inhibited 172 units/cc of needle callus glyoxalase I. The same volume of extract inhibited 10,290 units/cc of commercial enzyme by 92%.

Since the extract was strongly inhibitory to glyoxalase I, it was isoelectrically focused in hopes of removing the inhibitor. Previous focusing on Ampholine PAGplate gel had enabled glyoxalase I to be separated from most of the other protein. The extract was run on prefocused gel with a pH gradient from 3.5-9.5. The anode solution was 1M $H_3PO_4$ and the cathode solution was 1M NaOH. After two hours of focusing, a sample strip was removed from the gel for staining with MTT (36). The stain, specific for glyoxalase I, demonstrated glyoxalase I activity in the needle extract. To confirm this finding, the remainder of the gel fraction was extracted with 1.6 cc of 50 mM potassium phosphate buffer. This extract was assayed in the usual manner for glyoxalase I activity. Glyoxalase I activity was confirmed and quantitated at 45 units/g.f.w. Thus glyoxalase I is present in Douglas-fir needles and can be detected only after isoelectric focusing to remove the inhibitor.

## DETERMINATION OF MOLECULAR WEIGHT OF GLYOXALASE I

The glyoxalase I active extract from 17.82 g.f.w. of 31(DFN 94)11 after isoelectric focusing was dialyzed against dd $H_2O$. The remaining solution was freeze-dried and reconstituted as a 0.2% solution in 0.40 cc (0.25 cc of 0.05M $PO_4$, pH 6.8, and 0.15 cc of 0.20M NaCl). The sedimentation and diffusion coefficients were then determined for this solution as shown in Appendices VIII and IX. These values allowed a molecular weight of 18,220 to be calculated for the solution. Although the peak spread rapidly, indicating a polydisperse solution, the molecular weight is close to the value of 21,000 reported for a subunit of glyoxalase I by Uotila (26).

# CONCLUSIONS

Glutathione has been isolated and identified as the major nonprotein sulfhydryl component of Douglas-fir needle callus. Glutathione levels in the callus range from 0.08-0.40 μmoles per gram fresh weight. These levels are within the same range as those found in the yearly variation of spruce needles on a fresh weight basis. When subcultured on a monthly basis, the sulfhydryl levels tend to be high early in the period and low at the end of the period in needle callus.

For Douglas-fir needle callus clones 15 and 94, which have never produced shoots, the glutathione levels vary in an irregular fashion and the glutathione reductase activities do not correlate well with each other. Clone L-2, a shoot producing clone, shows good correlation between glutathione level and glutathione reductase activity as does the nonshoot producing clone 31.

Methylglyoxal has been isolated as its 2,4-dinitrophenylosazone from an insoluble fraction from Douglas-fir needles but was not observed in a similar Douglas-fir needle callus preparation. Methylglyoxal does have an inhibitory effect on the growth of Douglas-fir needle callus suspension cells. At $10^{-4}$M methylglyoxal inhibits growth by approximately 50%. Glutathione does not show any effect on growth as measured by dry weight increase at $10^{-4}$, $10^{-6}$, or $10^{-8}$M.

Douglas-fir needles contain methylglyoxal synthetase activity whereas Douglas-fir needle callus does not. This provides additional evidence for the validity of the isolation of methylglyoxal from Douglas-fir needles and not Douglas-fir needle callus. Methylglyoxal seems to play a very definite role in the development of Douglas-fir needles. In the young needles, cell division appears to be protected from the inhibitory braking action of methylglyoxal by methylglyoxal reductase. As the needle develops and reaches its

fully elongated stage the methylglyoxal reductase activity decreases and methylglyoxal synthetase activity peaks. This allows the buildup of methylglyoxal which could act as a brake on further cell division. As the needle matures, both of these enzymes are lost and the cells remain in a resting state. Douglas-fir needle development may involve the proper modulation of methylglyoxal reductase and synthetase activities and the inhibition of glyoxalase.

Neither glyoxalase I nor glyoxalase II activity was detectable in crude preparations from needle tissue. The needle extract, however, was found to strongly inhibit the glyoxalase I activity from needle callus as well as commercial enzyme. As little as 50 µL of needle extract, representing 1/100th of the total extract from 2.97 g of needles, totally inhibited 172 units/cc of needle callus glyoxalase I. The same volume of extract inhibited 10290 units/cc of commercial enzyme 92%. A glyoxalase I specific stain gave a positive test after isoelectric focusing of a needle extract. Buffer elution of the corresponding area of the gel gave a value of 45 units/g.f.w. of glyoxalase I. Douglas-fir needles thus contain glyoxalase I activity which is strongly inhibited in crude preparations. Isoelectric focusing separates the inhibitor from the enzyme allowing the observable activity.

The proliferative Douglas-fir needle callus cells meanwhile contain no methylglyoxal nor the enzyme to synthesize it. The results are consistent with cell division being unchecked by the brakelike action of methylglyoxal. Furthermore, any methylglyoxal entering the needle callus cells would be rapidly metabolized because of both methylglyoxal reductase and glyoxalase. Since needle callus tissue is the result of a wounding action, the presence of glyoxalase in needle callus is most easily viewed as a release from the inhibition found in

needles. With its lack of methylglyoxal and its strong complement of methylglyoxal degrading enzymes, the tissue is strongly proliferative by Szent-Gyorgyi's criteria.

## SUGGESTIONS FOR FUTURE RESEARCH

This research has established the presence of methylglyoxal in Douglas-fir needles. The enzymes capable of controlling its concentration in needles are modulated in a manner which is consistent with the idea that this substance may act as a cell division brake. In the developing needle it appears as if methylglyoxal may act as a cell division brake only after morphological patterns have been established. Thus, since plant hormones are considered the most likely regulators of differentiation, a study to observe the possible interaction between hormones and methylglyoxal-controlling enzymes might shed considerable light on growth and development in organized tissue. A key question to be answered here is whether or not expression of the enzymes like methylglyoxal reductase and synthetase are controlled directly by hormones or indirectly through inhibitors. The discovery of glyoxalase I activity from Douglas-fir needles, after isoelectric focusing, suggests the isolation and characterization of the inhibitor as a primary concern.

The situation in Douglas-fir needle callus relative to methylglyoxal metabolism can be characterized as strongly catabolic. The tissue is highly proliferative by Szent-Gyorgyi's criteria. The ability to directly assay a crude preparation for glyoxalase I activity indicates the lack of the inhibition demonstrated in needles. The tissue, by its nature, is obviously out of control hormonally. The need for synthetic auxins in spite of high levels of endogenous IAA demands further study. This work indicates that synthetic auxins may be necessary to keep phenolic compounds reduced and prevent the formation of toxic o-quinones. The thought that this may be mediated through the alternate respiratory chain of which glutathione and ascorbic acid oxidase are elements warrants further attention.

Needle callus browning, thought to occur by the buildup of phenolics, results in very low reduced glutathione levels. These observations have led to the possibility that the effect of synthetic auxin analogs is to cause an increase in reduced glutathione levels by inhibiting ascorbic acid oxidase. This idea is further reinforced by the finding of sufficiently high endogenous IAA levels in needle callus tissue.

Since the glutathione level does not closely follow glutathione reductase activity in clones 15 and 94, the masking effect is probably due to the synthesis of o-diphenols. Control of redox potential in these clones cannot occur then in the normal manner. Reducing power in the form of glutathione must be diverted to o-quinones to prevent their buildup and eventual browning of the tissue. Clones L-2 and 31 on the other hand, do not have as great a problem with phenolics and their redox potential can be regulated by more conventional metabolic events.

Not reported in the body of this thesis was a successful attempt at initiating callus without synthetic auxins. The combination of glutathione and IAA was sufficient to induce callus formation. The possibility exists here of producing callus free from possible synthetic auxin complications.

## GLOSSARY AND SPECIAL ABBREVIATIONS

ATP — 5'Adenosine triphosphate

Auxins — Plant growth hormones of several types which cause cell enlargement, apical dominance, and root initiation; a natural auxin is 3-indoleacetic acid

BAP — $N^6$-Benzylaminopurine; a synthetic cytokinin

Callus culture — Proliferation from a parental explant of many cells in protoplasmic continuity, but having no equivalence with any normal tissue

Cytokinin — A class of plant growth hormones associated with cell division; all have the $N^6$-substituted aminopurine unit in their structure

2,4-D — 2,4-Dichlorophenoxyacetic acid; a synthetic auxin

2,4-DNPH — 2,4-Dinitrophenylhydrazine

DTNB — Dithionitrobenzoate or Di(5-carboxy-4-nitrophenyl)disulfide; reagent used to assay free sulfhydryl groups

EDTA — Disodium ethylenedinitrilotetraacetate

g.f.w. — Grams fresh weight

$GSO_3$ — Oxidation state of glutathione after treatment with performic acid

IAA — 3-Indoleacetic acid; a naturally occurring auxin

Inhibitor — A substance which limits or destroys the catalytic activity of an enzyme

$K_m$ — Michaelis constant; substrate concentration at which $v = 1/2\ V_{max}$

MOPS — 3-(Morpholino)-propanesulfonic acid; a good buffer

MTT tetrazolium — [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl] tetrazolium bromide

NAA — Napthalene acetic acid; a synthetic auxin

S.E.M. — Standard error of the mean

I.U. — International Unit. Amount of enzyme required to catalyze the formation or decomposition of 1.0 micromole of substrate per min under optimal conditions.

ACKNOWLEDGMENTS

The author wishes to express his grateful appreciation for the valuable assistance rendered by the many people cooperating in this study. He is particularly indebted to his advisory committee: Dr. M. A. Johnson (Chairman), Dr. D. W. Einspahr, and Dr. L. L. Winton. It was Dr. Johnson whose constant guidance and generous help carried the entire work to its completion.

Many others made significant contributions to this research. The author wishes to thank Mrs. Shirley Verhagen for culturing callus, Mr. Russ Feirer for running the amino acid analyses, Mr. John Carlson for his expertise in laboratory technique, Mr. Fred Sweeney and Mr. Don Beyer for their photographic assistance, Dr. F. Skoog for supplying tobacco sources, M. Harder and G. Wyckoff for greenhouse assistance, and my mother Betty for typing the manuscript.

The financial support and educational opportunites offered by The Institute of Paper Chemistry were gratefully appreciated.

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# APPENDIX I

## CALLUS GROWTH MEDIUM

Calli were grown on Winton's Medium 11 the composition of which is listed in Table XII below. This represents the basal medium used to perpetuate Douglas-fir needle callus. The medium used to observe the effect of methylglyoxal and glutathione on growth contained no BAP and 1.0 ppm NAA. Tobacco crown gall medium contained no BAP or NAA.

### TABLE XII

### COMPOSITION OF WINTON'S MEDIUM 11

| Inorganic Nutrients | ppm | Organic Supplements | ppm |
|---|---|---|---|
| $NH_4NO_3$ | 1650 | Nicotinic Acid | 0.5 |
| $KNO_3$ | 1900 | Pyridoxine•HCl | 0.1 |
| $CaCl_2 \cdot 2H_2O$ | 440 | Thiamine•HCl | 0.1 |
| $MgSO_4 \cdot 7H_2O$ | 370 | Myo-inositol | 100 |
| $KH_2PO_4$ | 170 | Napthalene Acetic Acid | 5 |
| $H_3BO_3$ | 6.2 | $N^6$-Benzylaminopurine | 0.1 |
| $MnSO_4 \cdot H_2O$ | 16.9 | Sucrose | 30,000 |
| $ZnSO_4 \cdot 7H_2O$ | 10.6 | pH | 5.6 ± 0.2 |
| KI | 0.83 | | |
| $NaMoO_4 \cdot 2H_2O$ | 0.25 | | |
| $CuSO_4 \cdot 5H_2O$ | 0.025 | | |
| $CoCl_2 \cdot 6H_2O$ | 0.025 | | |
| Fe EDTA | 5.6 | | |

# APPENDIX II

## SAMPLE ENZYME UNIT CALCULATION

The following sequence shows a sample enzyme unit calculation used for glyoxalase I and II. Other enzymes were calculated in the same manner using the appropriate extinction coefficient.

$$\varepsilon_{240} = \frac{A}{\ell c}$$

where $\varepsilon$ = extinction coefficient

$A$ = absorbance

$\ell$ = path length in cm

$c$ = concentration in moles/liter

since $\ell = 1$ & $\varepsilon_{240} = 3370 \underline{M}^{-1}cm^{-1}$ for S-lactoylglutathione

then $\frac{A}{3370} \frac{\text{moles}}{\text{liter}} \div \text{time} = \frac{\text{moles}}{\text{liter} \cdot \text{min}} \times 1 \times 10^6 = \frac{\mu\text{moles}}{\text{liter} \cdot \text{min}}$

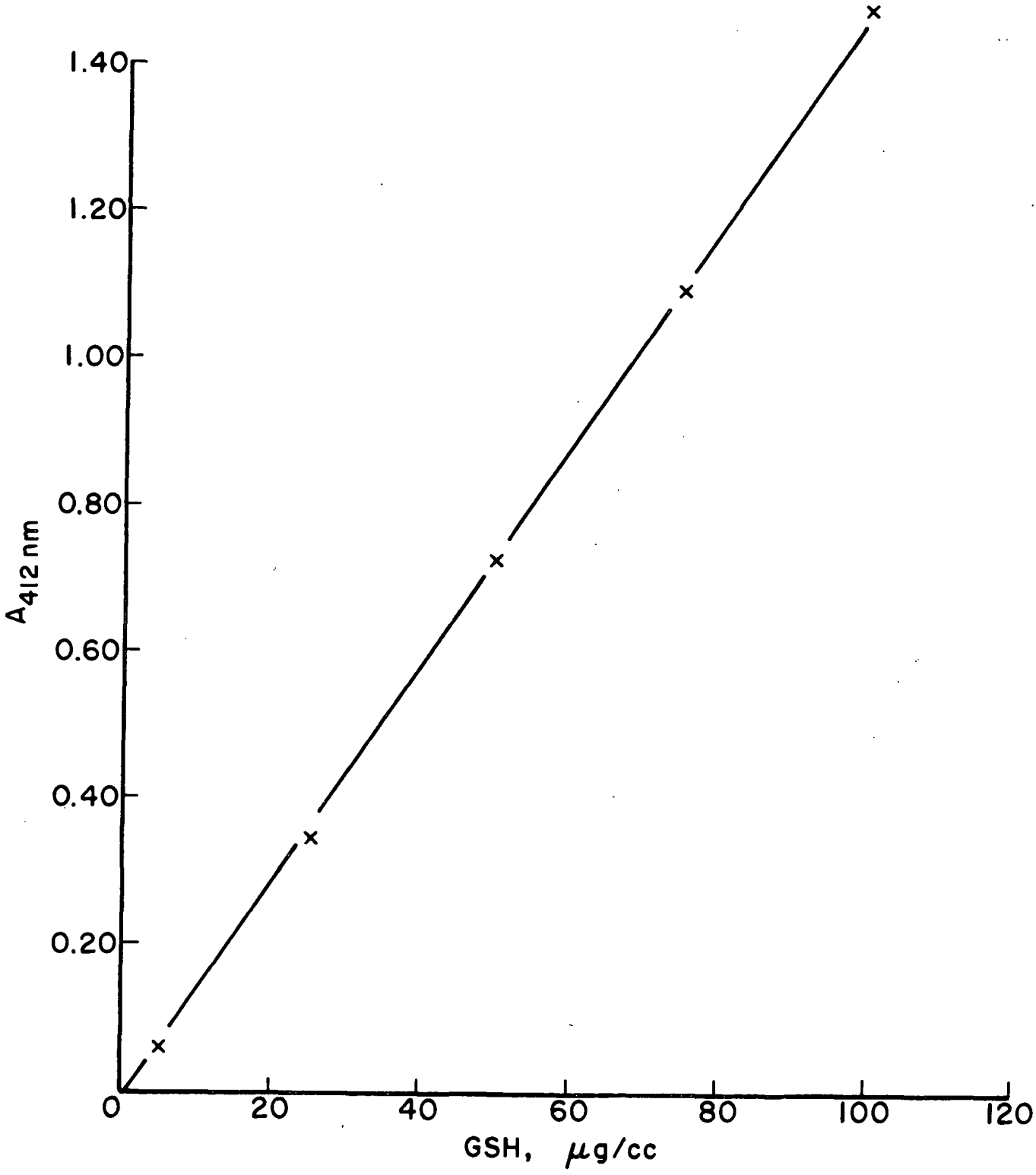
$\div 1000 = \frac{\mu\text{moles}}{\text{cc} \cdot \text{min}} \times \text{final volume} = \frac{\mu\text{moles}}{\text{min}} = \text{units/0.1 cc of enzyme solution}$

units/0.1cc x 10 = units/cc of enzyme solution x total volume = total I.U.

x 1 x $10^3$ = units as $\frac{\text{nmoles}}{\text{min}}$

APPENDIX III

NONPROTEIN SULFHYDRYL CALIBRATION CURVE
DETERMINED SPECTROPHOTOMETRICALLY USING DTNB REAGENT



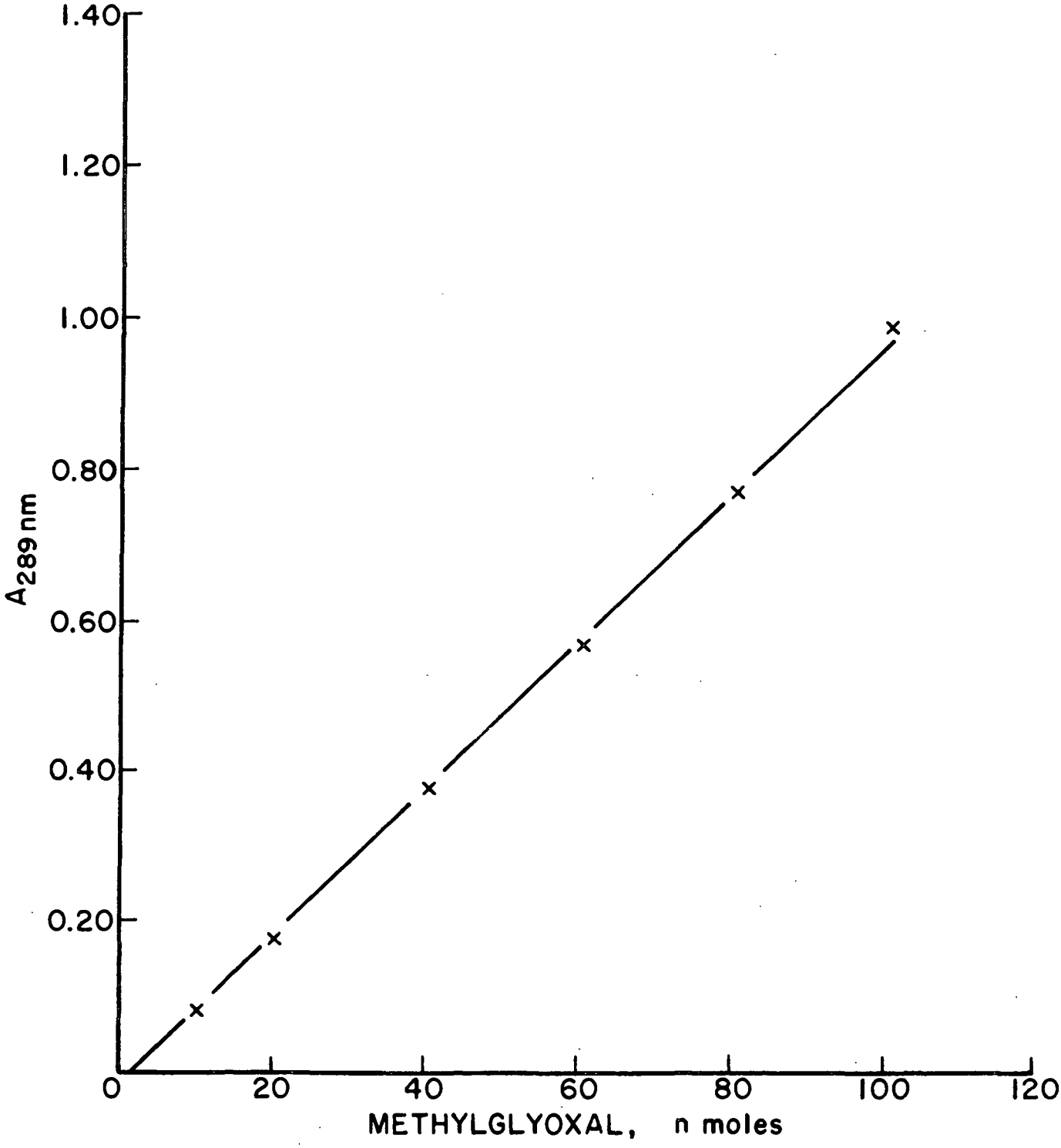
# APPENDIX IV

## NONPROTEIN SULFHYDRYL AND CORRESPONDING GLUTATHIONE REDUCTASE DATA

| Sample | Weeks Since Subculture | Average SH µgrams/g.f.w. | S.E.M. | GSH Reductase units/g.f.w. |
|---|---|---|---|---|
| 6(L-2)11 | 1 | 75.03 | 0.3 | 3.7 |
| | 2 | 76.59 | 1.7 | 39.2 |
| | 3 | 63.85 | 0.8 | 39.1 |
| | 4 | 38.54 | 0.6 | 30.9 |
| | 1 | 124.11 | 1.1 | 92.8 |
| | 2 | 77.27 | 1.5 | 127.2 |
| | 3 | 47.93 | 0.9 | 64.2 |
| | 4 | 19.27 | 1.2 | 19.6 |
| 10(DFN 15)11 | 1 | 93.83 | 1.4 | 37.5 |
| | 2 | 89.21 | 3.3 | 7.7 |
| | 3 | 34.21 | 2.6 | 16.6 |
| | 4 | 22.94 | 1.4 | 13.0 |
| | 1 | 87.24 | 1.4 | 27.9 |
| | 2 | 43.34 | 1.6 | 33.3 |
| | 3 | 57.94 | 1.3 | 69.2 |
| | 4 | 54.83 | 0.8 | 58.0 |
| 25(DFN 31)11 | 3 | 111.73 | 1.2 | 63.8 |
| | 4 | 67.13 | 1.8 | 19.1 |
| | 1 | 34.64 | 0.6 | 11.4 |
| | 2 | 87.38 | 1.3 | 44.6 |
| | 3 | 62.96 | 1.0 | 92.0 |
| | 4 | 22.87 | 2.7 | 36.6 |
| | 1 | 96.72 | 0.9 | 26.6 |
| | 2 | 69.93 | 0.3 | 61.3 |
| 26(DFN 94)11 | 1 | 67.35 | 0.9 | 10.9 |
| | 2 | 75.49 | 1.2 | 32.7 |
| | 3 | 28.30 | 2.3 | 38.2 |
| | 4 | 27.87 | 11.2 | 66.0 |
| | 1 | 11.83 | 1.2 | 52.6 |
| | 2 | 43.56 | 1.6 | 159.6 |
| | 3 | 60.02 | 1.2 | 71.6 |
| | 4 | 67.69 | 0.7 | 144.3 |

APPENDIX V

METHYLGLYOXAL CALIBRATION CURVE DETERMINED
SPECTROPHOTOMETRICALLY USING GIRARD T REAGENT



# APPENDIX VI

## INFLUENCE OF METHYLGLYOXAL ON GROWTH OF DOUGLAS-FIR CELL SUSPENSIONS

| Sample | Time in Days Since Start of Experiment | Average Dry Weight, mg | S.E.M. |
|---|---|---|---|
| Control | 0 | 30.2 | 1.6 |
| MG $10^{-4}$ | 0 | 25.7 | 2.0 |
| MG $10^{-6}$ | 0 | 23.1 | 1.9 |
| MG $10^{-8}$ | 0 | 26.9 | 0.9 |
| Control | 2 | 29.1 | 0.2 |
| MG $10^{-4}$ | 2 | 26.0 | 2.0 |
| MG $10^{-6}$ | 2 | 29.2 | 1.7 |
| MG $10^{-8}$ | 2 | 22.5 | 2.8 |
| Control | 4 | 31.9 | 2.4 |
| MG $10^{-4}$ | 4 | 29.7 | 0.9 |
| MG $10^{-6}$ | 4 | 26.4 | 1.0 |
| MG $10^{-8}$ | 4 | 22.7 | 1.5 |
| Control | 6 | 21.6 | 1.3 |
| MG $10^{-4}$ | 6 | 22.1 | 1.3 |
| MG $10^{-6}$ | 6 | 21.8 | 1.0 |
| MG $10^{-8}$ | 6 | 23.8 | 0.8 |
| Control | 8 | 29.4 | 1.2 |
| MG $10^{-4}$ | 8 | 27.4 | 1.8 |
| MG $10^{-6}$ | 8 | 29.5 | 1.0 |
| MG $10^{-8}$ | 8 | 22.6 | 0.8 |

APPENDIX VI (Continued)

INFLUENCE OF METHYLGLYOXAL ON GROWTH OF DOUGLAS-FIR CELL SUSPENSIONS

| Sample | Time in Days Since Start of Experiment | Average Dry Weight, mg | S.E.M. |
|---|---|---|---|
| Control | 10 | 32.1 | 1.7 |
| MG $10^{-4}$ | 10 | 21.0 | 1.1 |
| MG $10^{-6}$ | 10 | 32.5 | 2.0 |
| MG $10^{-8}$ | 10 | 29.9 | 1.0 |
| Control | 14 | 45.3 | 1.6 |
| MG $10^{-4}$ | 14 | 24.9 | 1.5 |
| MG $10^{-6}$ | 14 | 42.3 | 0.6 |
| MG $10^{-8}$ | 14 | 38.0 | 1.4 |
| Control | 17 | 54.0 | 0.6 |
| MG $10^{-4}$ | 17 | 32.5 | 1.4 |
| MG $10^{-6}$ | 17 | 49.9 | 1.0 |
| MG $10^{-8}$ | 17 | 49.7 | 1.1 |
| Control | 24 | 83.7 | 0.6 |
| MG $10^{-4}$ | 24 | 40.3 | 2.0 |
| MG $10^{-6}$ | 24 | 79.5 | 2.4 |
| MG $10^{-8}$ | 24 | 66.7 | 1.2 |
| Control | 32 | 112.0 | 3.4 |
| MG $10^{-4}$ | 32 | 59.0 | 1.4 |
| MG $10^{-6}$ | 32 | 130.8 | 2.0 |
| MG $10^{-8}$ | 32 | 116.7 | 7.1 |

## APPENDIX VII

### INFLUENCE OF GLUTATHIONE ON GROWTH OF DOUGLAS-FIR CELL SUSPENSIONS

| Sample | Time in Days Since Start of Experiment | Average Dry Weight, mg | S.E.M. |
|---|---|---|---|
| Control | 0 | 30.2 | 1.6 |
| GSH $10^{-4}$ | 0 | 25.9 | 2.0 |
| GSH $10^{-6}$ | 0 | 23.1 | 1.3 |
| GSH $10^{-8}$ | 0 | 25.3 | 2.6 |
| Control | 2 | 29.1 | 0.2 |
| GSH $10^{-4}$ | 2 | 25.8 | 1.5 |
| GSH $10^{-6}$ | 2 | 25.5 | 2.5 |
| GSH $10^{-8}$ | 2 | 24.5 | 1.7 |
| Control | 4 | 31.9 | 2.4 |
| GSH $10^{-4}$ | 4 | 26.4 | 2.5 |
| GSH $10^{-6}$ | 4 | 28.2 | 2.6 |
| GSH $10^{-8}$ | 4 | 29.3 | 2.2 |
| Control | 6 | 21.6 | 1.3 |
| GSH $10^{-4}$ | 6 | 24.5 | 1.3 |
| GSH $10^{-6}$ | 6 | 27.0 | 1.0 |
| GSH $10^{-8}$ | 6 | 18.6 | 1.0 |
| Control | 8 | 29.4 | 1.2 |
| GSH $10^{-4}$ | 8 | 21.9 | 0.5 |
| GSH $10^{-6}$ | 8 | 22.2 | 0.6 |
| GSH $10^{-8}$ | 8 | 21.8 | 0.5 |

APPENDIX VII (Continued)

INFLUENCE OF GLUTATHIONE ON GROWTH OF DOUGLAS-FIR CELL SUSPENSIONS

| Sample | Time in Days Since Start of Experiment | Average Dry Weight, mg | S.E.M. |
|---|---|---|---|
| Control | 10 | 32.1 | 1.7 |
| GSH $10^{-4}$ | 10 | 29.4 | 1.0 |
| GSH $10^{-6}$ | 10 | 31.9 | 0.2 |
| GSH $10^{-8}$ | 10 | 29.2 | 0.3 |
| Control | 14 | 45.3 | 1.6 |
| GSH $10^{-4}$ | 14 | 39.6 | 1.7 |
| GSH $10^{-6}$ | 14 | 43.8 | 3.2 |
| GSH $10^{-8}$ | 14 | 34.6 | 1.7 |
| Control | 17 | 54.0 | 0.6 |
| GSH $10^{-4}$ | 17 | 50.1 | 1.5 |
| GSH $10^{-6}$ | 17 | 53.9 | 0.6 |
| GSH $10^{-8}$ | 17 | 54.5 | 1.0 |
| Control | 24 | 83.7 | 0.6 |
| GSH $10^{-4}$ | 24 | 78.7 | 1.2 |
| GSH $10^{-6}$ | 24 | 89.1 | 2.3 |
| GSH $10^{-8}$ | 24 | 74.7 | 1.4 |
| Control | 32 | 112.0 | 3.4 |
| GSH $10^{-4}$ | 32 | 118.1 | 0.8 |
| GSH $10^{-6}$ | 32 | 109.5 | 4.4 |
| GSH $10^{-8}$ | 32 | 117.1 | 2.3 |

# APPENDIX VIII

## SEDIMENTATION COEFFICIENT OF GLYOXALASE I

The sedimentation coefficient at 25°C in 0.05M $PO_4$, pH 6.8, and 0.20M NaCl for glyoxalase I from Douglas-fir needles was determined by John Carlson. The log sheet for this determination is on file at The Institute of Paper Chemistry (Beckman Model E, Log Sheet No. 1904). The plot from which the sedimentation coefficient was obtained is presented in Fig. 29.

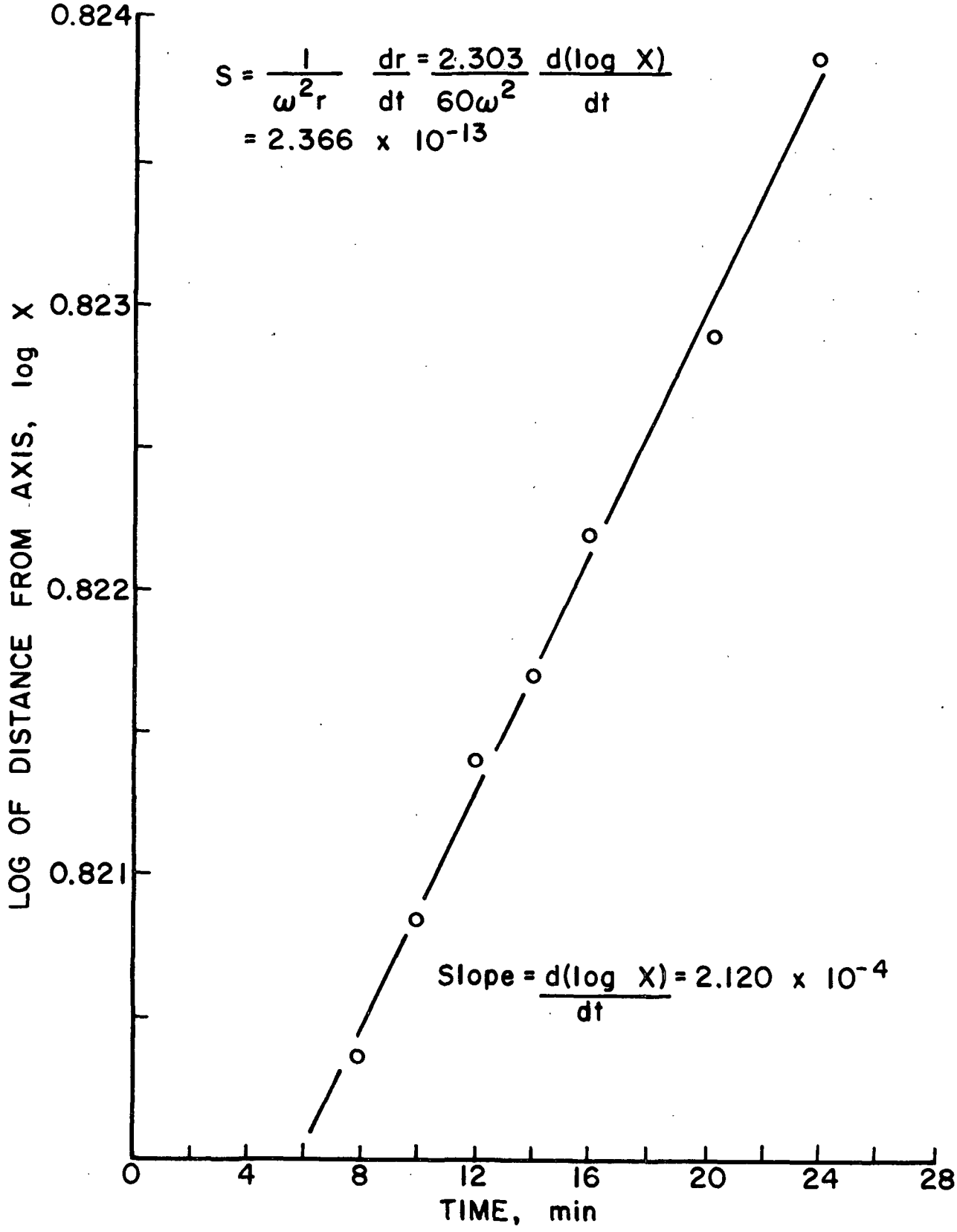



Figure 29. Determination of Sedimentation Coefficient

# APPENDIX IX

## DIFFUSION COEFFICIENT OF GLYOXALASE I

The diffusion coefficient at 25°C in 0.05M $PO_4$, pH 6.8, and 0.20M NaCl for glyoxalase I from Douglas-fir needles was determined by John Carlson. The log sheet for this determination is on file at The Institute of Paper Chemistry (Beckman Model E, Log Sheet No. 1905). The plot from which the diffusion coefficient was obtained is presented in Fig. 30.

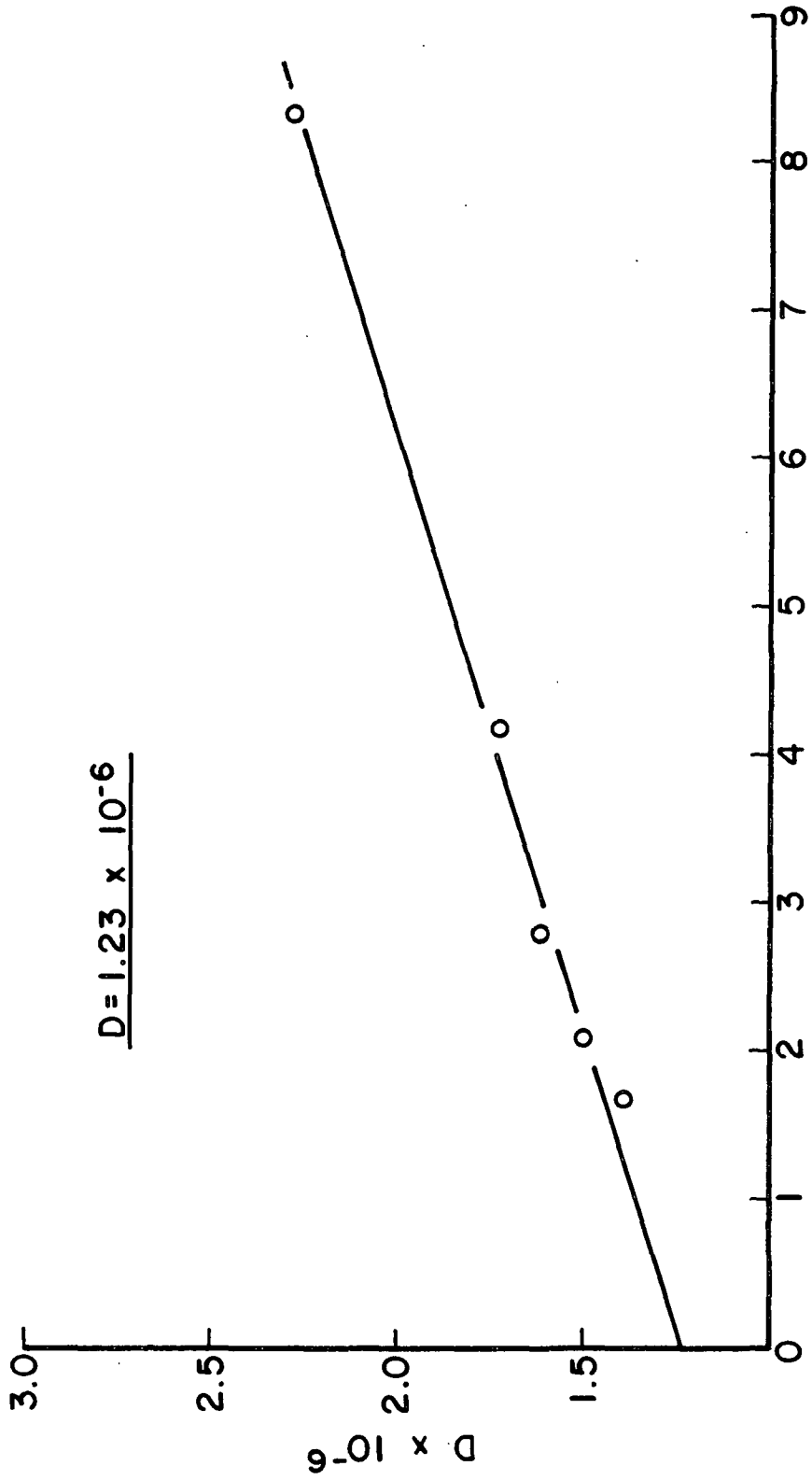



Figure 30. Determination of Diffusion Coefficient

# APPENDIX X

## MOLECULAR WEIGHT CALCULATION OF GLYOXALASE I

$$M = \frac{RTs}{D(1-\bar{v}\rho)}$$

where M = molecular weight

R = universal gas constant

T = absolute temperature

s = sedimentation coefficient

D = diffusion coefficient

v = partial specific volume(assumed)

$\rho$ = solvent density

$$M = \frac{(8.314\times10^{7})(2.98\times10^{2})(2.37\times10^{-13})}{(1.23\times10^{-6})(0.262)}$$

$$= 1.822\times10^{4}$$

$$M = 18{,}220$$

# APPENDIX XI

## PROTEIN ASSAYS

Protein levels were determined by the microbiuret method (122). The reagent was prepared by combining 25.0 cc of 28.5-30.0% $NH_3$, 1.25 g $CuSO_4 \cdot 5\ H_2O$, 230 cc 10M NaOH and diluting to 500 cc with dd $H_2O$. The sample cuvette contained 2.0 cc of reagent, 0.1 cc of sample, and 0.9 cc of 50mM phosphate buffer, pH 6.8. The reference cuvette contained 2.0 cc of reagent and 1.0 cc of phosphate buffer. Sample absorbances were read 30 minutes after mixing at 330 and 392 nm. The protein concentration was determined with a standard calibration curve constructed with bovine serum albumin. This curve is shown in Fig. 31.

Although many protein assays were conducted on various Douglas-fir needle as well as needle callus samples, no such data is presented in the body of the thesis. Significant correlations between protein levels and various other factors could not be made. This data can be found in Notebook No. 3262.

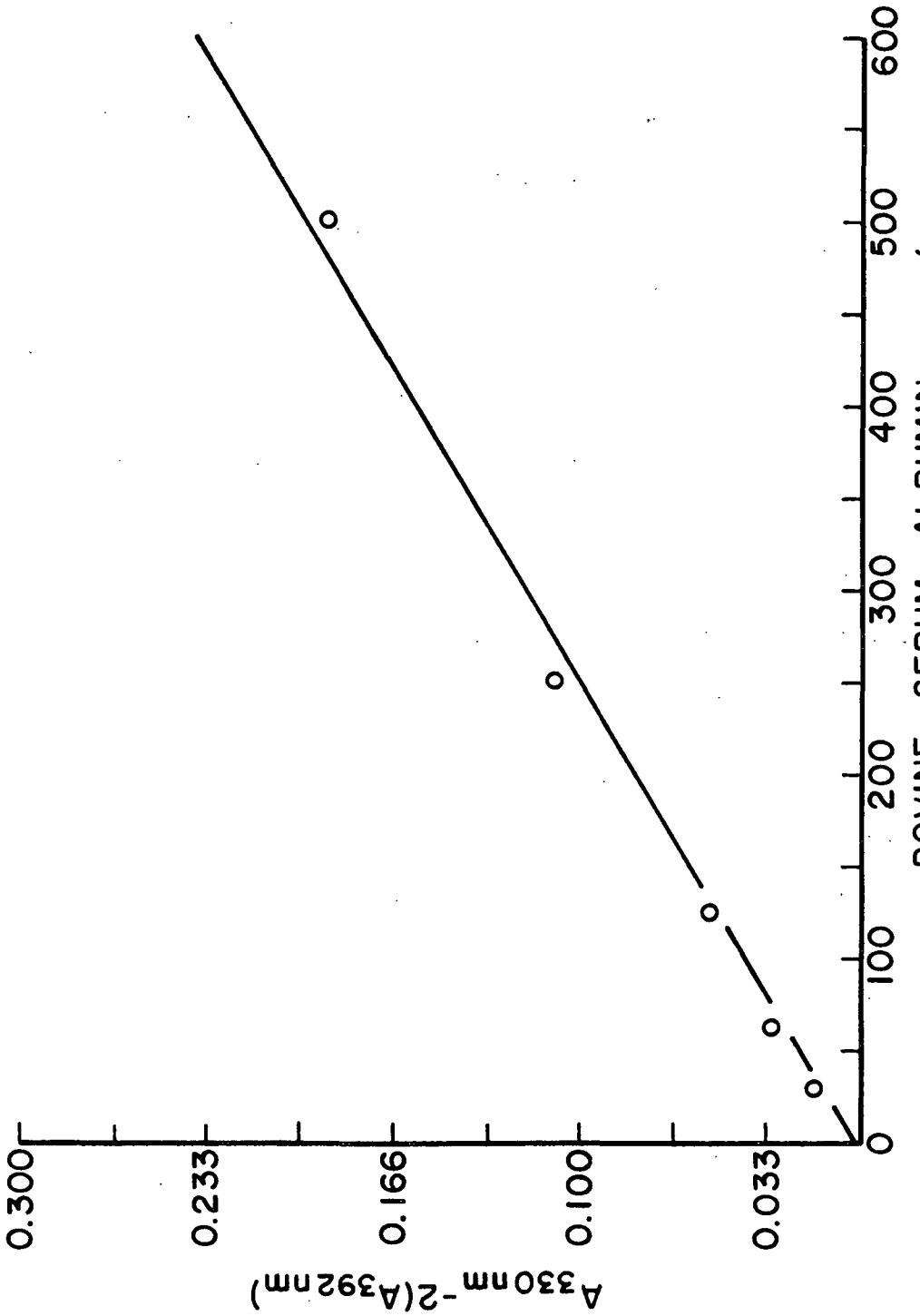



Figure 31. Protein Calibration Curve

# APPENDIX XII

## DNA AND RNA ASSAYS

The method of Cherry (123) was used for the extraction and assay of nucleic acids. The DNA content was determined with diphenylamine reagent according to the procedure of Giles and Myers (124). This value was then subtracted from the total nucleic acid value to give the RNA concentration. The assay involves absorbance readings at 595 and 700 nm after solution incubation for 16 hours at 30°C in the dark. The sample cell contained 493 μmoles of diphenylamine reagent, 2.9 μmoles of acetaldehyde and 2.0 cc of sample. The reference cell contained 493 μmoles of diphenylamine reagent, 2.9 μmoles of acetaldehyde and 2200 μmoles of $HClO_4$. Both solutions were in a total volume of 3.0 cc. The reagent was 4.0% diphenylamine in fresh glacial acetic acid. The DNA concentration was determined from a calibration curve of μg DNA/cc *versus* absorbance at 595-700 nm. The standard curve is shown in Fig. 32.

Similar to the protein assays, many DNA and RNA assays were conducted on various samples. Lack of significant correlations between nucleic acid levels and various other factors is the reason that no such data is presented in the thesis. This data can be found, however, in Notebook No. 3262.

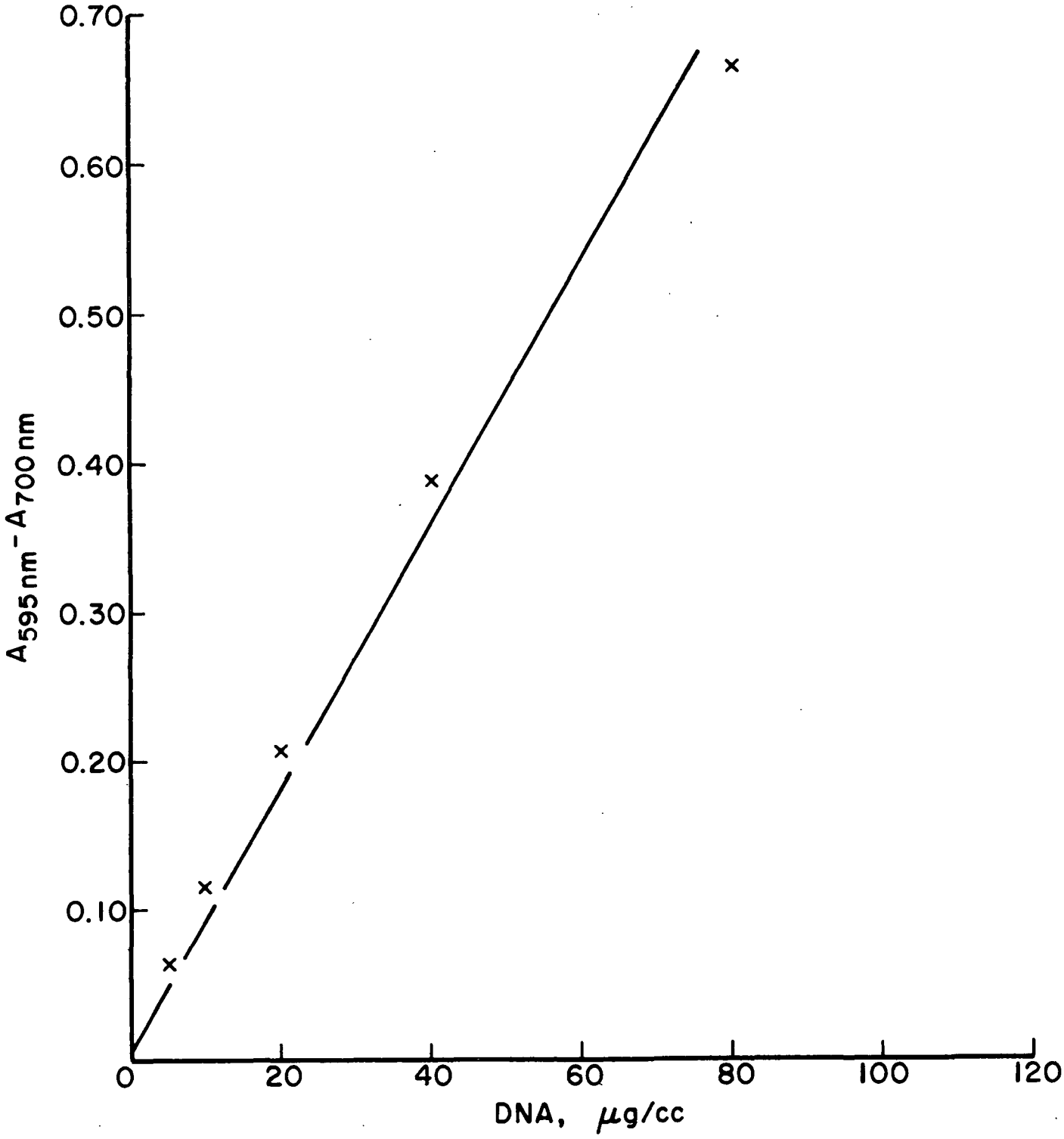



Figure 32. DNA Calibration Curve